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EDITOR'S NOTE

Owing to the lack of appropriation no volume of Collected Studies has been issued from this laboratory since 1919. During the interval the majority of the articles in the present volume have been published elsewhere and with some revision are reprinted here from the various medical journals in which they originally appeared.

With three exceptions, no papers have been included in the present volume which were written later than the end of 1926.

In certain cases, for the sake of brevity and more systematic arrangement, several papers on the same topic have been combined to form one article, and most articles have been abbreviated, with reference to the original source of publication.

INDEX OF AUTHORS

Appelbaum, Emanuel 229, 241, 251, 264

Banzhaf, Edwin J...77, 113, 121, 123, 126, 133, 144, 210, 212, 367, 501, 528
Behner, D. 364
Berman, Fannie 364
Bittman, Florence R.........148, 321, 393, 399, 527
Blanc, Ninette E.................. 153

Carmichael 364
Cooper, Georgia M.....153, 163, 173, 436, 449, 457, 468, 475

Denny, Helen T.................. 166

Elmendorf, Ten Eyck.............. 279

Forbes, Mary V.................. 552
Freeman, Roland G., Jr........... 384

Gerber, Herman 494, 528
Goldman, Agnes 160, 506, 508, 515
Gosling, Ruth 274
Greenwald, Charles K........113, 121, 144
Gross, Louis 204
Grund, Marie 380, 468, 475
Gurley, Caroline R......148, 325, 364, 489

Hatfield, Hazel M................ 531
Hazen, Elizabeth Lee............. 527
Hillman, Oliver 298
Honig, Florence 563
Hussey, Helena 148, 160
Hyslop, George H................ 298

Jackson, Henry W...........229, 251, 260
Jones, Archibald 311

Kolchin, Betty S................204, 364
Koopman, John 570, 580
Kraus, Walter M.................. 298
Krumwiede, Charles ...145, 196, 201, 420, 435, 436, 449, 457, 461, 468, 475, 486, 494, 499, 501, 503, 529

LaMont, Anna C. 166

Mann, Alice G. 82, 148
Martin, M. Edward................ 483
Mishulow, Lucy 153, 184, 191, 420, 429, 483, 503
Montanus, John 274

Neal, Josephine B......215, 217, 221, 229, 241, 247, 251, 260, 266, 279, 282, 298, 311, 314
Nedley, Rose M.................. 563
Nevin, Mary 148, 321, 393, 399, 527
Noble, W. Carey................435, 529
North, Charles E................. 541

Oldenbusch, Carolyn 420, 429

Park, William H.......13, 27, 59, 66, 77, 82, 89, 123, 145, 173, 177, 327, 360, 361, 384, 386, 468, 475, 531, 541
Parker, Julia T................... 212
Platou, E. S...................... 95
Povitzky, Olga R...166, 402, 409, 452, 455
Provost, Dorothy J.....436, 457, 494, 166

Rosenstein, Carolyn 468, 475

Salthe, Ole 461
Scholl, Marie 429
Schroeder, Mae C.........32, 59, 89, 123
Schwartz, Florence 552
Shapiro, Louis L.................. 282
Spiegel, Rose Goldschmidt.......... 361

Tyler, Charles H............468, 475, 486

Unneberg, Aarlang 160

Valentine, Eugenia..163, 184, 196, 199, 201
Von Sholly, Anna I..........177, 183, 428

Weinberg, Jacob 520, 526
Wilcox, Harriet Leslie...........105, 135
Williams, Anna W..82, 145, 148, 160, 325, 360, 364,386, 402
Wilson, M. A...............386, 552, 563

Zingher, Abraham.......36, 42, 50, 66, 77, 123, 342, 368

INDEX OF SUBJECTS

PAGE

- Agglutinins
  - A note on the claim that they are lipoidal in nature 435

- Agglutinin
  - Absorption 436

- Anaerobic Bacillus
  - See "Food Infection" 483

- Antibodies
  - (See hemolytic streptococci) 325

- Antiserums
  - (See antitoxins) 501

- Antitoxin
  - Diphtheria
    - A study of methods of administration 95
    - Protective value of standard monovalent antitoxin 82
    - A new method of adding cresol 501
  - Scarlatinal
    - Complexity 361
    - Purification 367
    - Treatment 327
  - Staphylococcus
    - Production in horses 212

  - Cholera-Suis (See Food Infection) 457

- Bacillus
  - Coli (See meningitis) 266
  - Diphtheria
    - Agglutination characteristics 82
    - The effect of peptone on the toxigenic property of 105
  - Influenzae
    - Multiplicity of races as determined by agglutination and agglutinin absorption 163
    - Grouping of Influenza bacilli with special reference to permanence of types 166
    - Accidental inoculation 173
    - Growth without hemoglobin 402
  - Paratyphosus
    - (See meningitis) 264
    - of rodent origin 461
  - Pertussis
    - Methods of cultivation 409
    - Immunological types 420
  - Tetani
    - The effect of peptone upon the production of toxin 135

  - Tuberculosis
    - Direct cultivation from spinal fluids of human and bovine types 274

- Botulinus toxin
  - (See immunization) 528

Complement fixation PAGE
  In chronic gonorrhoea in women 552
  Hemolytic titration; limits of accuracy 570
  Osmotic pressure and its relation to specific and non-specific hemolysis 580

Cresol
  (See antitoxins) 501

Dick Test
  In normal persons and in acute and convalescent Scarlet fever 342

Diphtheria 13, 27, 32, 42, 50, 59, 66, 82, 89, 95, 113, 123, 126, 133

Dropping Bottle
  An aid in macroscopic slide agglutination 499

Encephalitis
  Epidemic 221, 229
  Associated with measles 241

Erysipelas
  (See hemolytic streptococci) 364

Ferry Diplococci
  (See measles) 386

Food Infections
  Due to a paratyphoid bacillus of rodent origin 461
  Due to tapioca pudding contaminated with B. cholerae suis 457
  An anaerobic bacillus presumably the cause of a fatal infection 483

Frost Little Plate Method
  In routine milk examination 531

Gold Chloride Reaction
  A colorimetric reaction apparently indicative of toxic strength 503

Gonorrhea
  Complement Fixation in chronic cases in women 552

Hemolysis
  Limits of accuracy in titration 570
  Osmostic pressure and its relation to specific and non-specific hemolysis 580

Hydrogen
  Ion concentration 520

Hypersensitiveness
  To whole horse serum or serum globulins, etc 89

Immune Bodies
  Cross Protective power of anti-pneumococcus monovalent serums Type I, II and III 204
  Distribution in pneumococcus serum Types I, II and III 210
  Relationship between different antibodies of hemolytic streptococci from Scarlet fever 325

Immunization PAGE
  Against Botulinus toxin 528
  Against diphtheria 13, 27, 32, 42, 59, 66, 123
  Results with Scarlatinal toxin 342

Intestinal Bacteriology 506, 508, 515

Kahn Precipitation Method 563

Measles
  Encephalitis associated with measles 241
  Convalescent whole blood, plasma and serum in prophylaxis 368, 384
  Susceptibility of rabbits to the virus 380
  Relationship of Tunnicliff and Ferry diplococci to measles 386
  Experimental measles in rabbits and monkeys 393, 399

Meningitis
  Conditions noted during the epidemic of influenza 215
  Distribution according to age and etiology 247
  Epidemic 251, 260
  Influenzal 217
  Caused by B. coli 266
  Caused by meningococci and paratyphoid bacilli 264
  Spontaneous hemorrhage 314
  Sporotrichosis 298
  Streptococcic 311
  Streptothrix 279
  Torula 282
  Tubercle bacilli—Direct cultivation from spinal fluids 274

Milk
  (See Frost Little Plate method) 531
  (See pasteurization) 529, 541

Oysters
  Effect of storage and changing sea-water on contaminated oysters 468
  Chlorine treatment of contaminated oysters 475

Pasteurization
  On the claim that some typhoid-paratyphoid strains survive milk pasteurization 529
  Standards for 541

Peptone
  Effect on toxin production 105, 135

Pertussis
  Vaccination as a prophylactic measure 428

Phosphate Broth
  Elimination of precipitate 526

Pneumococci
  Study of serological relationship; from common colds and influenzal conditions 153

Poliomyelitis
  Experimental in rabbits 321

Precipitation PAGE
Nature and value of the precipitin reaction in the serological grouping of streptococci ... 196

Precipitins
Antibacterial specificity of absorption ... 449

Ramon
Flocculation test in diphtheria ... 452
Flocculation test in scarlet fever ... 455

Respiratory infection ... 145, 148, 153, 160, 177, 184, 191, 196
Microbal studies of immunological types ... 145
Methods of demonstrating microorganisms and filterable viruses, etc. ... 148
Relation of alpha type streptococci to ... 160
Prophylactic vaccination against ... 177
Hemolytic streptococci from upper respiratory tract ... 184

Scarlet Fever
Etiology, prevention by immunization and antitoxic treatment ... 327
Dick Test ... 342
Antitoxin purification ... 367
Serum of dual potency ... 360
Complexity of scarlatinal toxin ... 361
Ramon flocculation test ... 455
Streptococci ... 325, 364

Schick Test
Degree of immunity to diphtheria insured by a negative Schick test ... 27
Accuracy of reaction ... 36
In schools ... 50

Serum
Pneumococcus
Cross protective power of types I, II, III ... 204
Distribution of immune bodies in types I, II, III serums ... 210
Antiscarlatinal ... 360, 361

Sporotrichosis
Meningitis ... 298

Staphylococcus
(See antitoxin) ... 212

Streptococcus
Alpha type; relation to upper respiratory infections ... 160
Hemolytic; cultural and serological relationship ... 184
Hemolytic; A study of various methods of demonstrating hemolysis ... 191
Hemolytic; In Scarlet fever and Erysipelas ... 364
Loss of hemolytic capacity without change in agglutination characteristics ... 199, 201
Meningitis following Scarlet fever ... 311
Relationship between different antibodies of hemolytic streptococcus from Scarlet fever ... 325
Non hemolytic from human intestinal tract ... 515
Precipitin reaction and serological grouping ... 196

Streptothrix
Meningitis ... 279

PAGE

Syphilis
- Kahn precipitation method in diagnosis 563

Torula
- Meningitis 582

Toxin Antitoxin
- Diphtheria
  - Immunization against diphtheria 13
  - Duration of immunity 32
  - Results obtained in schools 66
  - Comparative merits of new and old preparations 59
  - New Mixture (1/10 L +) 77
  - The effect of freezing 113
  - Preparation of mixture 121, 133
  - Decrease of immunizing value when stored one year 126
  - Hypersensitiveness following injections 89
- Tetanus
  - Active immunization in guinea pigs 144

Toxoid
- Diphtheria
  - As an immunizing agent 66, 123

Tuberculosis
- Unsuccessful attempts to cure or prevent in guinea pigs with Dreyer's defatted antigen 527

Tunnicliff Diplococci
- Relation to measles 386

Vaccination
- Against acute respiratory diseases 177

Vaccine
- Pertussis
  - Potency of stored vaccines 429
  - As a prophylactic measure in Pertussis 428
- Pneumonia
  - Therapy for Pneumonia 183

Vaccine Virus
- Danger of decolorizing 486

Vaccinia 489

Virus
- Susceptibility of rabbits to virus of measles 380
- Malignant disease among poultry 494

DEATH OF DR. ANTHONY

The death of one of the most valued members of our staff, Dr. Bertha Van Houton Anthony occurred in 1923 from pneumonia.

Because of her marked administrative ability she was put in charge of the Division of Media and Sterilization, a division which she helped to organize and which she conducted successfully up to the time of her death. In connection with this work she was given the office title of Chief of Division.

Dr. Anthony had a remarkable ability to express scientific facts in clear and correct English. For a number of years she assisted in editing the Collected Studies and of late had entire charge of this arduous and exacting task. In doing this work, which required so much time and patience, she rendered an invaluable service to all members of the laboratory staff. We are indebted to Dr. Anthony for the idea of general editorial supervision and in no line of work will she be more keenly missed than in this.

TOXIN-ANTITOXIN IMMUNIZATION AGAINST DIPHTHERIA*

William H. Park, M.D.

The development of antitoxin in the bodies of animals injected with diphtheria toxin is one of the best established of facts.

Unaltered diphtheria toxin has been used also as a stimulating injection in man, but it has to be used in very small and repeated doses; otherwise it would produce annoying local reactions and possibly even paralysis. It was early realized that if we ever attempted actively to immunize children, the advantage would be great if we could replace pure toxin with an altered toxin which would be equally effective and without danger.

Use of Toxin Modified by Antitoxin for Active Immunization in Animals

The earliest knowledge that injections of toxin almost neutralized by antitoxin are capable of stimulating in animals the production of antitoxin came quite accidentally. As is well known, antitoxin can be detected and measured only by its characteristic of neutralizing toxin. The degree of neutralization of the toxin by the antitoxin is determined by the injection of a definite quantity of the mixture into guinea-pigs. The testing of the potency of the drawings of the serum from the various horses under treatment is likely to leave a certain proportion of the test animals alive because of their having received subcutaneously toxin with an overneutralizing amount of antitoxin. The attempt to use these treated guinea-pigs several months later revealed the fact that many of the animals were immune. Investigation proved that this antitoxin immunity did not appreciably develop until the lapse of from four to six weeks.

Babes, in 1895, was the first to inject experimentally diphtheria toxin-antitoxin mixtures and to appreciate that not only slightly underneutralized diphtheria toxin, but also that which was slightly overneutralized, would bring about the development of antitoxin in animals.

A little later (1896), but independently, I made the same observations. Wernicke, in 1895, noted that guinea-pigs actively immunized by the injection of living bacilli and antitoxin gave birth to immune young, and that antitoxin was present in both the mothers and their offspring for at least eight months. In the winter of 1896, I began to use this knowledge concerning much overneutralized toxin practically, in starting the immunization of the horses which were employed for the production of diphtheria antitoxin.

I noted that the use of large amounts of overneutralized toxin was a safe and rapid method of beginning immunization. In the same year, Dreyer and Madsen made systematic investigations as to the effect of different degrees of overneutralization of the effectiveness of the toxin-antitoxin to stimulate antitoxin.

In 1903 I published results showing that an injection into a horse of 10,000 lethal doses of toxin which has been just neutralized usually causes the development of about 60 units of antitoxin per cubic centimeter of serum, while the same toxin neutralized sixfold usually causes the production of about three units.

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*Published in The Journal of The American Medical Association, 1922, Vol. LXXIX, pp. 1584-1590.

In 1905, Theobald Smith studied the duration of immunity in guinea-pigs which had received toxin-antitoxin. He corroborated the work of Wernicke, and extended to two years the time during which guinea-pigs retain immunity.

The fact that the antitoxin would take a number of weeks to develop in those not originally possessing it made it not applicable to persons in immediate danger of infection. Therefore, in the presence of diphtheria, immunizing antitoxin injections would still be necessary in families and institutions. In the absence of any simple test for determining which individuals had natural antitoxin and which did not, we had to inject many unnecessarily, if active immunization were to be attempted. The success of the treatment would also be difficult to determine. These considerations delayed its application in man. On the other hand, we had the growing conviction that, wonderful as were the results of antitoxin, diphtheria could never be conquered by it. The combination of the ever present carrier of bacilli, and the slowness of people to recognize diphtheria when it develops, still permits the occurrence of 50 per cent. more diphtheria than we would otherwise have, and perhaps one sixth of the number of deaths. Furthermore, vital statistics revealed that diphtheria mortally and morbidity had not decreased during the last five years; for instance, in the state of New York, the mortality was 12.8 in 1917 and 16.8 in 1921.

Only one means presented itself to our minds which gave any hope of making another distinct advance. This was active immunization by means of toxin injections.

In 1913, the application by Schick of the intracutaneous neutralization test to detect antitoxin immunity gave a simple means of testing for antitoxic immunity before and after the injections.

Practical Application of Diphtheria Toxin-Antitoxin in Man

Von Behring, May 8, 1913, reported the injections of neutralized toxin in a small number of persons. Before giving the injections he used no Schick tests or other means of testing whether or not the subjects were already immune. He demonstrated by repeated guinea-pig tests that there was a quick development of antitoxin in many of those treated, but in others no antitoxin was detected at the time the tests were made. It is now known that the retests were made too soon to detect the development of antitoxin in those who had none at the time of the injection. The duration of the immunity was not investigated. Although von Behring referred to the toxin-antitoxin mixture as his discovery, what he used was exactly what several of us had described and used in experimental immunization during the last eighteen years. His real contribution was the demonstration in a few human beings of the safety of the injections.

The example of von Behring led Hahn and Summer shortly afterward to offer the toxin-antitoxin to the 4,300 children of six villages in the district of Magdeburg, where diphtheria was endemic. Of the 1,097 children children injected, 633 received the full series of three injections; 225 received two injections, and 209 received one. The Schick test was not used before or after treatment, so it is impossible to know how many of the injected children possessed natural antitoxin and how many of the others developed it.

There was no difference in the development of cases of diphtheria among the treated and the untreated portion during the first two weeks following the completion of the injections; but after that time there was a lessening of the number in the treated portion. The immunization had no apparent effect on freeing carriers from infection. Until after the war, no further observations or immunizations were made in Europe.

Oct. 21, 1920, Bieber published his study of the histories of all the children mentioned above. Fortunately, he was able to trace the majority of those who were living in the district at the time of the injections. He reported that during the six years, 15 per cent. of those who had not received injections had had attacks of diphtheria; that only 4.6 per cent. of the total number inoculated, and only 3.3 per cent. of the fully treated had developed it. Because of the war, many of the diagnoses were not confirmed by cultures, and he believed that some of the cases were almost surely not diphtheria. Two of the incompletely treated children had died from diphtheria.

Bieber concluded that although the toxin-antitoxin had not given a lasting immunity to all who had received the injections, the results were satisfactory in that the percentage of persons having diphtheria was very much less in the inoculated portion than in that uninoculated. He believed that the results should stimulate more extensive trials.

PERSONAL OBSERVATIONS

Before and since the period of the war, the practical value of the toxin-antitoxin injections was subjected to continuous investigation by workers in the Bureau of Laboratories of the Department of Health of the City of New York. The results obtained and the conclusions drawn as follows:

THE ANTITOXIN RESPONSE AND THE PERMANENCE OF THE IMMUNITY ACQUIRED

Late in 1913 we began the practical use of toxin-antitoxin injections for the immunizing of children against diphtheria, and established the facts that the procedure was harmless and that after three injections about 80 per cent. of those individuals possessing no antitoxin or insufficient antitoxin to protect from diphtheria developed immunity. Those showing positive Schick reactions and receiving two injections, developed negative Schick reactions in about 70 per cent.; those receiving one injection, in about 50 per cent. We soon realized that the most important problem was the duration of the antitoxic immunity in those that had developed anti-toxin. A satisfactory answer to this question required that immunizations be carried out in institutions where the children would be under observation for a number of years. A few suitable institutions were immediately sought for and obtained by Dr. Abraham Zingher and, later, additional ones were added by Dr. M. C. Schroeder. We have thus been able to keep under supervision for from three to six and one-half years about 10,000 children. From year to year, Drs. Zingher and Schroeder have been reapplying the Schick test to these original children. With a few of them we are now in the seventh year of observation. We have had no serious immediate or late after-effects. In these institutions diphtheria has not developed in any child who has received three injections. Eighty per cent. of those who received three inoculations have developed sufficient antitoxin within three months to prevent the positive Schick reaction. Fifty per cent. of the remainder developed antitoxic immunity sufficient to give the negative Schick test before the end of the first year. The remainder received then or later a second series of injections, and all of these concerning whom we have information became immune. In some later investigation, we have encountered occasional children who resisted even two series of injections.

Discussion of Results of Tests

As an example of the results following the injections in the school-children, Table 1 is given.

The knowledge that about 80 per cent. of the children who possess no diphtheria antitoxin develop it after three injections of toxin-antitoxin and with very few exceptions retain it for at least six years, and that those who partially fail to respond do respond after a second series of treatments, affords us ground for the belief that we have a practical means of immunizing the child population of the country. Even though the belief that this change in the antitoxin content of these children is a permanent one should prove erroneous, it would merely mean that one would have to repeat the injections not oftener than once in six years.

TABLE 1.

RESULTS OF ATTEMPTED IMMUNIZATION AS SHOWN BY SCHICK TEST THREE OR MORE MONTHS AFTER TREATMENT WITH THREE INJECTIONS OF TOXIN-ANTITOXIN; CHILDREN FROM PUBLIC SCHOOLS OF BROOKLYN; TESTS BY DR. SCHROEDER

Number of School	Location	Total Number Children Retested	Positive Cases Which Became Immune	Per Cent.	Length of Period Between Treatment and Retest
158	Belmont Ave. near Warwick St...	370	254	68.7	3 mos.
156	Sutter Ave. near Barrett St......	329	292	88.8	3 mos.
173	Penn. Ave. near Liberty Ave.....	163	144	88.9	4 mos.
29	Columbia and Amity Sts..........	57	45	79.0	5 mos.
142	Henry and Rapelye Sts...........	127	112	88.0	5 mos.
50	S. 3d St. and Driggs Ave.........	82	74	90.3	6 mos.
16	Wilson Ave. near Bedford Ave...	241	216	89.7	6 mos.
72	New Lots Road.................	199	195	98.0	6 mos.
109	Dumont Ave. near Sackman St...	141	99	70.0	6 mos.
4	Berkeley Pl. near 5th Ave........	103	91	88.4	7 mos.
	Total	1,812	1,522	83.9	

Permanence of Negative Schick Reaction in Persons Who Develop Natural Immunity

At the Convent of St. Dominick, ninety-four of the original children who had given Schick negative reactions with Dr. Zingher's tests remained for five years. These children were retested at the two and five year periods by Dr. Schroeder. In both retests ninety of these children showed negative reactions, while four showed positive reactions in one or the other retest. It is an interesting point as to whether the children who were negative originally had lost their antitoxin or whether the apparent change in reaction was due to other causes. We know from making the double Schick test (that is, one test on each arm) on several hundreds of children, that routine tests made even by an expert are likely to show occasional errors. In this test series, about 2 per cent. of the children showing positive tests had them only on one arm. There was, therefore, no doubt that in 2 per cent. of these children, one of the two injections had been inserted too deeply so that the toxin did not remain in the skin and therefore could not produce the reaction. We know also that slight differences in the strength of the toxin solution cause a borderland case to give either a negative or a positive reaction. Differences in technic and in toxin solution possibly account, therefore, for the apparent change in one or all of the four cases. On the other hand, we know that the amount of antitoxin in an individual changes somewhat from time to time, so that it is possible that one or all of the four subjects might have reacted at one time and not at another to the standard Schick toxin properly given.

Whatever the explanation of the apparent change in four cases, we have the remarkable fact that nearly 96 per cent. of the originally negative children remained negative during five years. Practically the same results have been obtained in all the other institutions.

INFLUENCE OF AGE ON SUSCEPTIBILITY AND THE RESPONSE TO IMMUNIZING INJECTIONS

It is common belief that the mortality from diphtheria is greatest at the ages of from one-half year to 4 years, inclusive; that it then drops steadily until at 10 years it is quite low and so remains during the rest of life. The figures in Table 2 give evidence that this belief is founded on fact.

TABLE 2.

DEATHS FROM DIPHTHERIA GROUPED BY SEX AND AGES, CITY OF NEW YORK, YEAR 1917

Age	Males	Females	Total	Per Cent.
Under 1 year	73	60	133	11+
1 to 4 years inclusive	390	319	709	60+
Total under 5 years	463	379	842	72+
5 to 9 years inclusive	119	132	251	21+
10 to 14 years inclusive	9	13	22	2
15 to 19 years inclusive	6	4	10	1
20 to 24 years inclusive	2	8	10	1
25 to 60 years inclusive	9	14	23	2
Total all ages	608	550	1,158	

The greater liability to infection during the first years of life is clearly shown in the results from the Schick test. Our findings in New York City are given in Table 3.

All reports show that under similar conditions the highest percentage of persons having antitoxin occurs among infants passively carrying their mothers' antitoxin, and among adults; between the ages there is first a decrease and then an increase. The same reports show that similar age groups of different children reveal surprising differences in the percentages of those possessing antitoxic immunity.

TABLE 3.

AVERAGE SUSCEPTIBILITY OF VARIOUS AGES TO DIPHTHERIA (AS INDICATED BY THE POSITIVE SCHICK DIPHTHERIA-TOXIN SKIN TEST), IN NEW YORK CITY*

Age	Schick + (Susceptible)
Under 3 months	15 per cent.
3 to 6 months	30 per cent.
6 months to 1 year	60 per cent.
1 to 2 years	60 per cent.
2 to 3 years	60 per cent.
3 to 5 years	40 per cent.
5 to 10 years	35 per cent.
10 to 20 years	25 per cent.
20 to 40 years	18 per cent.
Over 40 years	12 per cent.

*The percentage of positive reaction is considerably higher among those living in the country.

Table 4 notes the very small percentage of susceptibles in an institution caring for little children gathered from the poorest and most crowded homes of the city. Table 5 exhibits the surprising differences in the results obtained from tests of pupils in different parts of Brooklyn.

TABLE 4.

RESULTS OF THE SCHICK TEST IN AN INSTITUTION CARING FOR CHILDREN GATHERED FROM DENSELY POPULATED PORTIONS OF NEW YORK CITY

	Shick Reactions				
	Positive		Borderland	Negative	Per Cent. Positive
Age	+	±	±	—	
Under 5 years	6	2	0	35	18.5
5 to 10 years	21	2	8	220	9.0
10 to 15 years	5	2	4	79	7.7
	32	6	12	334	9.9

The sign ± indicates a trace of antitoxin.

A minimum, an average and a maximum result from among the larger Brooklyn public schools subjected to the Schick test by Dr. Schroeder or her assistants is given in Table 5. The ages ranged from 6 to 12 years.

TABLE 5.

DIFFERENT PERCENTAGES OF CHILDREN GIVING SCHICK-POSITIVE REACTIONS IN FOUR LARGE PUBLIC SCHOOLS

No. of Brooklyn School	Total Number of Tests	Per Cent. Positive
50	1,433	19.60
125	1,064	36.84
149	1,056	41.19
156	1,148	61.84

The highest percentage of positive tests obtained by Dr. Schroeder in any Brooklyn school was 72, and the lowest 16. These were in small schools. The average percentage of positives among 30,000 children in seventy-three Brooklyn schools was 41.5.

In the Manhattan schools at the same time or a little later, Dr. Zingher among 52,000 children found that the percentage of positives was 30. The highest percentage of positives in any school was 67, and the lowest, 13.6.

The results obtained by Dr. Schroeder among adults are very interesting as showing a much older age group. Among 4,396 adult inmates in an insane asylum there were 633 positive reactions, a percentage of 16. At all ages between 20 and 50 years, the percentage was about the same, but above 50 years of age the positives dropped to 10. There were a good many persisting pseudoreactions which made it extremely difficult in some instances to decide between a pseudoreaction and a combined reaction. The patients responded to the toxin-antitoxin injections as well as younger people, except that they showed more local reaction.

Immunizing Effect of Toxin-Antitoxin Injections in Infants at Birth and During the First Two Years of Life

If it were possible to immunize young infants, this would be most desirable. In order to test this possibility, 2,000 infants were given full doses by Miss Denny on the third, eighth and eleventh days after birth. Most careful observations revealed absolutely no deleterious effects. At the end of a year, 100 of these infants were retested. Only 52 per cent. gave negative Schick reactions. Since untreated infants of this age give about the same result, it is evident that the combined effect of immature cells and the overneutralization of the toxin-antitoxin present (because of the passive immunity derived from the mother) prevents any appreciable response at birth to the toxin-antitoxin injections. Infants aged 6 months and over gave far better results.

Thus Dr. Blum observed the results of the injections in a number of older infants, in the Home for Hebrew Infants. His figures are given in Tables 6 and 7.

Dr. Byard also reports very favorable results among children in private homes. Many of these were not given the Schick test before receiving the immunizing injections. It is fair to assume that about 50 per cent. would have given negative Schick reactions. Of 286 such children, more than half

TABLE 6.

RESULTS OF TOXIN-ANTITOXIN INJECTIONS IN SCHICK-POSITIVE CHILDREN

Number of Infants	Age	Toxin-Antitoxin Injections	Lapsed Period at Time of Retest	Results of Shick Test
6	5 mos.	3	3-5 mos.	—
5	5 mos.	3	9 mos.	—
3	6 mos.	3	4 mos.	—
4	7 mos.	3	4-5 mos.	—
12	8 mos.	3	4-5 mos.	—
Total 30				

TABLE 7.

RESULTS OF TOXIN-ANTITOXIN INJECTIONS IN SCHICK-NEGATIVE INFANTS

Number of Infants	Age	Toxin-Antitoxin Injections	Lapsed Period at Time of Retest	Results of Schick Test
1	3 mos.	3	4 mos.	±
2	4 mos.	3	4 mos.	—
2	5 mos.	3	4 mos.	—
3	6 mos.	3	4 mos.	—
6	7 mos.	3	4 mos.	—
1	8 mos.	3	4 mos.	—
1	9 mos.	3	4 mos.	—
3	10 mos	3	4 mos.	—
1	11 mos.	3	4 mos.	—
Total 20				

of whom were under 1 year of age when injected, he noted that 143 (under 2 years of age when injected) when retested after seven months showed 136 negative, five positive and two very doubtful Schick reactions. Of the whole number, seven, or 2.4 per cent., when retested at the end of eight months were definitely positive. Eighteen months after the injections, 1.8 per cent. were positive. These results are certainly very encouraging.

These results of Blum and Byard among infants aged 4 months and over are of extreme practical importance, because from 6 months to 3 years is the period when immunization is most necessary and when it creates the least disturbance.

Constitutional and Local Reactions Following the Toxin-Antitoxin Injections

Constitutional and local reactions following the toxin-antitoxin injections are negligible in the infant; slight and infrequent in the young child; moderate or rather severe in perhaps 10 per cent. of older children, and slight, moderate or quite severe in a large percentage of adults. The effects are largely due to the protein contents of the culture fluid and are not due to the toxin as such. This is evident because the same reaction follows the injection of the toxin broth rendered atoxic by heating, or of a solution containing a minute quantity of autolyzed diphtheria bacilli. If the toxin were the cause, there would be little or no reaction in naturally immune persons. As is well known, these show fully as much reaction as those who have no antitoxin, if not more reaction. Those individuals who give the strong pseudoreactions with the heated or unheated toxin of the Schick test are those who give the most severe reactions with the toxin-antitoxin injections. However, some who give no pseudoreaction with the Schick test give moderately severe reactions to the toxin-antitoxin injections. The horse serum is present in such a minute amount as to cause no appreciable reaction except in a few extremely susceptible individuals. It does not seem to sensitize them appreciably to later doses of horse serum.

In children of school age about 10 per cent. develop fairly sore arms and temperature of from 99 to 103 F. About 5 per cent. feel miserable enough to stay at home from school for one day, and a very few for two days. We have given about 40,000 inoculations this fall and winter without a single infection. Children that are constipated are advised to take a laxative on the day of the injection and to apply a moist dressing to the arm if swelling and soreness develop.

In adults, the reactions are not quite as severe as with the typhoid inoculations. The most severe reactions are restricted almost entirely to those who develop the marked pseudoreaction with the heated or overneutralized toxin. The following history gives an account of one of the most severe reactions that we have encountered:

A nurse at the Willard Parker Hospital was given toxin-antitoxin subcutaneously in the right arm, February 8. In the evening the arm in the region of the injection felt sore and looked red. The next day the soreness and tenderness were more marked. The highest temperature was 103 F. February 9, the patient was in bed. The temperature was 99. The right arm showed moderate redness of the lower two thirds, some induration and tenderness, and slight axillary tenderness. February 10, constitutional symptoms were absent. The temperature was normal. Redness and swelling persisted; induration was moderate; tenderness and pain were less marked; axillary tenderness was very slight. A Schick test and control which were made, February 9, both showed a moderate pseudoreaction, the two reactions appearing alike. An area of redness about the size of a 25 cent piece and some induration were present. A small secondary areola around the central area was of marked redness. The probability is great that the test was a pseudoreaction and not a combined reaction.

The Toxin-Antitoxin Mixture

The best mixture is one that is underneutralized and yet perfectly safe. As the mixture stands, the toxin deteriorates faster than the antitoxin, so that it gradually becomes neutralized and later overneutralized by the antitoxin.

TABLE 8.

RESULTS OF INJECTION OF DIFFERENT PREPARATIONS OF TOXIN-ANTITOXIN IN GUINEA-PIGS

Preparation											
Number	Amt. in C.c.	Number Doses	Weeks Elapsed	Percentage of Neg. Schick Reactions	Number Doses	Weeks Elapsed	Percentage of Neg. Schick Reactions	Number Doses	Weeks Elapsed	Percentage of Neg. Schick Reactions
24	0.5	1	7	100	2	5	100	3	7	100
18	0.5	1	12	66	2	4	75	3	9	100
22	0.5	1	8	50	2	8	50	3	7	100
20	0.5	1	..	..	2	9	33	3	9	25
24A*	0.5	1	..	..	2	7	50	3	7	50
24	0.1	3	7	75	4	7	100			
18	0.1	3	10	50	4	9	60			
22	0.1	3	8	25	4	8	25			
20	0.1	3	8	0	4	8	40			
24A*	0.1	3	7	0	4	8	0			
10	0.1	3	8	0	4	8	0			

*Preparation of 24 with an added 10 per cent. of its original amount of antitoxin.

Therefore, a safe preparation is at its best when freshly prepared. As it would not be practicable to make new mixtures every day, we keep both the toxin and the antitoxin until they have become stabilized. After this has occurred, the toxin-antitoxin mixture changes very slowly. Even so, it is better not to use mixtures later than three months after their preparation unless animal tests prove them to be still slightly underneutralized.

It is noticeable that Preparation 24 gave much better immunizing than any of the others, except Preparation 18, which was a little inferior. The addition of 10 per cent. of the original amount of antitoxin to Preparation 24 lessened greatly its value. The other preparations had stood for some time, become less toxic and lost in immunizing power.

The comparative results of animal and human inoculations with different preparations show that very considerable differences in immunizing value exist. As yet we find it extremely difficult to determine by animal tests the finer differences which decide just when the ideal degree of neutralization is obtained. Dr. Banzhaf now standardizes our preparation so that 1 c.c. causes paralysis in the majority of guinea-pigs weighing 250 gm.; in all, 5 c.c. causes paralysis which ends in death. The results obtained in children and in animals with five different preparations clearly bring out the different degrees of success which are obtained with unequally neutralized preparations (Tables 8 and 9).

TABLE 9.

IMMUNIZING EFFICIENCY OF DIFFERENT MIXTURES OF TOXIN-ANTITOXIN IN CHILDREN

School or Institution	Mixture Three Doses 1 C.c. Each	Date Injected	Date Retested	Total Number of Children	Results +	Results —	Percentage of Positive Reactions
Colored Orphan Asylum	No. 18	12/ 5/19	3/ 1/20	70	3	67	95.7
Hebrew Sheltering and Guardian Home	No. 22	4/24/20	10/23/20	84	27	57	70.2
N. Y. Orphanage	No. 22	6/ 5/20	10/23/20	63	35	28	44.4
P. S. No. 90	No. 24	12/17/20	5/ 3/21	160	20	140	87.5

SAFETY OF TOXIN-ANTITOXIN INJECTIONS

In utilizing a substance which is a combination of a poison and an antipoison, it is of extreme importance to be certain that there is no possibility of a properly neutralized mixture becoming at some later period toxic. We have tested out many mixtures and kept them at all possible temperatures, and always found that the mixtures became very slowly more and more overneutralized. The antitoxin portion of the combination is more stable than the toxin. The only objection to using products that have been kept a long time after neutralization is that they are somewhat less effective. The only accident that has occurred in the use of toxin-antitoxin mixtures, so far as is known, was due to an error through which an improperly tested product was sent out. The regulations now adopted by all biologic plants make a repetition of such an accident impossible.

Practical Application in the Public Schools

The health and school authorities determined to put their chief efforts for the first two years into an attempt to immunize as many schoolchildren as possible rather than endeavor to protect the children of preschool age who needed the protection so much more. The reasons for this were many and important. It was deemed wise to acquaint as many parents and others as possible with the value of the Schick test and the toxin-antitoxin injections. No better way seemed available than to use the schools as the means of doing this. If each pupil presented his parents with a circular picturing the danger from diphtheria, describing the preventive treatment and asking for permission to administer this treatment if the family physician approved, it would mean that nearly a million adults and a million children would have the arguments for the use of the toxin-antitoxin vaccine presented to them in a favorable way. If, as we hoped, about one half of the children brought back the acceptance of the offer to give them the toxin-antitoxin, we should be able not only to immunize those shown by the Schick test to possess no antitoxin but, by preventing those children from contracting diphtheria, we should also prevent their carrying diphtheria home to the younger children in their families.

The testing of the schoolchildren would also give us the chance to determine exactly what effect the immunization had had. We could make an index of 90,000 children who had been tested and, when Schick positive, had been injected with toxin-antitoxin; a similar index of 90,000 children of the same ages who had refused the test and the injections could be used for comparison. The cases of diphtheria occurring among the children of school age could thus be looked up in either index, and observations made as to how many cases of diphtheria occurred in the two groups. We believed that after one or two years of such observations we should have had evidence of convincing value as to the protection guaranteed by a negative Schick test due either to natural antitoxic immunity or to the response to the toxin-antitoxin injections.

Now, at the end of two years, we actually have such an index completed. The 90,000 tested children and the 90,000 control children have been gathered about equally from the schools of the different boroughs; they were collected by the groups of nurses and teachers from the schools which had been tested by Dr. M. C. Schroeder and Dr. Abraham Zingher. Among an equal number of untreated and treated children, there were four times as many cases among the untreated children. The majority of the cases in both groups were of slight or moderate severity. Some of them were possibly cases of nondiphtheritic tonsillitis in children who happened to be carriers of diphtheria bacilli. The methods which we have adopted in the diphtheria prevention work in the schools have already been published by Dr. Zingher (No. 96 of the Reprint Series, Department of Health, City of New York). I will describe them briefly.

A very important point is the enlistment of the interest of the principal of a school. As a rule Dr. I. H. Goldberger, of the bureau of educational hygiene of the department of education, first prepares the way by obtaining permission for us to do the work in the school. According to the borough in which the school is situated, Dr. Zingher, Dr. Schroeder or one of their representatives sees the principal and explains fully the objects we have in view. Literature is left for the teachers. Either the principal or the physician meets the teachers in a conference, gives them the necessary information, and tries to arouse their enthusiasm. The success or failure in getting a favorable response from the children or their parents depends largely on the interest which the principal, the assistant principal and the teachers take in the matter. When they give us their enthusiastic cooperation, we expect to obtain consents from three fourths of the parents; when we fail to arouse the teachers' interest, we are fortunate if this is obtained from one fourth of them.

The preparatory work being furnished and the date for the test being determined, we send to the school the circulars and consent slips to be taken by the children to their homes.

Table 10 shows the results among the 180,000 schoolchildren whom we have listed.

TABLE 10.

NUMBER OF CASES OF SUSPECTED DIPHTHERIA DEVELOPING IN 90,000 UNTREATED SCHOOLCHLDREN AND IN 90,000 TESTED BY SCHICK METHOD AND WHEN POSITIVE GIVEN TWO OR THREE INJECTIONS OF TOXIN-ANTITOXIN

	Cases Reported as Clinical Diphtheria
In Brooklyn:	
26,000 originally Schick-negative children (observation from October 1 to February 15)	2
15,000 Schick-positive children, 3, 2 or 1 toxin-antitoxin injections	4*
40,000 control children of same ages	27
In Manhattan:	
31,000 Schick-negative children (observation from October 1 to February 15)	3
19,000 Schick-positive children, 3, 2 or in injections	5
50,000 control children	29
Summary:	
57,000 Schick-negative children (observation from October 1 to February 15)	5
33,000 Schick-positive children injected with toxin-antitoxin	9
Among a total of 90,000 Schick-negative or injected children	14
Among a total of 90,000 control children untreated	56

*Two of these children received but one injection and had no Schick retest. The third showed a Schick + and the fourth a Schick ± reaction on the retest and refused further injections.

As may be seen, there were among the 90,000 untreated schoolchildren four times as many cases of probable diphtheria as developed in the 90,000 tested children.

Summary and Conclusions

The results of twenty-five years of experimental and practical investigation of the immunizing effect of toxin-antitoxin injections and the value of the Schick test are as follows:

Three injections, 1 c.c. each, of a suitable toxin-antitoxin mixture spaced one or two weeks apart, will cause about 85 per cent. of susceptible children or older persons to develop sufficient antitoxin to give the negative Schick reaction and produce marked, if not absolute, protection against diphtheria.

The development of the immunity is slow. An amount of antitoxin sufficient to prevent the positive Schick reaction develops in the different children in from one to six months after the receiving of the injections. Antitoxin as heretofore must continue to be used to produce immediate immunity.

The duration of the immunity in at least 90 per cent. of the children is for more than six years and probably for the remainder of life. There seems to be no difference in this respect between these, and those who develop antitoxin naturally.

Toxin-antitoxin injections should not be given within two weeks after an injection of antitoxin; otherwise the toxin is slightly overneutralized and the resulting development of antitoxin is lessened.

Mixtures made from old toxin and antitoxin are fairly stable and may be used for a period of one year. Each such preparations are at their best when first sent out, as the mixtures slowly tend to become at first neutralized and then slightly antitoxic. This change gradually lessens the immunizing power of the toxin. The toxin-antitoxin should be kept cool and in a dark place; it is best to use the mixtures within three months after their final preparation.

A toxin-antitoxin mixture of stabilized materials which is safe when it leaves the laboratory cannot become more toxic on being kept. No serious effects have ever resulted from the injections given to the tens of thousands of the New York children since we began our work, seven years ago.

The Schick test is an extremely reliable means of separating those individuals who have antitoxic immunity from those that have none. Although a simple test, it must be carried out with extreme care. The toxin must be retained intracutaneously, and the toxin must be neither 25 per cent. more nor less than the desired amount. It is extremely important to choose glass of suitable chemical composition for the containers in which the toxin is to be placed, as otherwise rapid deterioration may take place.

The preliminary Schick test is usually omitted in children under 3 years of age. This is for two reasons: 1. Two thirds of these children require the toxin-antitoxin injections anyway. 2. We are not certain whether those that do give the negative reaction are immune because of an unusual persistence of the antitoxin given them by their mothers, or because of the active development of antitoxin in their own bodies. After this age the test is desirable, but it is often omitted.

Thus, in practical school work, the first Schick test is frequently omitted in children up to the age of 6 because it is easier to inject the children at once rather than to delay for the test. At this age the percentage of children requiring immunization is still high, and the annoyance from the injections is slight. The omission of the preliminary Schick test facilitates the introduction of the immunizing injections in the schools. Above the age of 6 years, the preliminary Schick test should be made whenever practicable.

No child should be pronounced immune from diphtheria because of having received three immunizing injections of toxin-antitoxin. A negative Schick test is absolutely necessary before one can properly make such a statement or issue a certificate.

The use of the control protein test made with the heated toxin is advisable at all ages when a careful separation of the pseudonegative reaction from the combined positive reaction is important. As with the Schick test, it is frequently omitted because of local conditions. The older the child the more likely it is to be immune and to give a confusing protein reaction.

In children under 5 years of age, the protein reaction seldom confuses the picture if the Schick tests are read as late as on the fourth day; between 5 and 7 years, the control does not help greatly in more than 5 per cent. In older children and adults, not only does the control protein test help us to decide more correctly in about 10 per cent. of doubtful reactions; but when it is marked it also indicates with some probability those persons who are likely to have the marked local and constitutional reactions from the toxin-antitoxin injections.

The toxin-antitoxin injections are inadvisable before the age of 6 months. During this time most of the infants retain the antitoxin received from their mothers. Up to the age of 3 months, immunizing injections are usually ineffective, as the infant tissues do not respond sufficiently during this period to the toxin-antitoxin to produce antitoxin. Under usual conditions, it is probably safe to wait until the infant is 9 months old and then to give the injections at the first suitable occasion. During the first three years there is almost no annoyance from the injections. As the child grows older, the danger from diphtheria gradually lessens, and the percentage of those developing annoying local and constitutional reactions slowly increases.

The immunization of schoolchildren in acting to prevent their contracting diphtheria also lessens the exposure to infection of the younger children of preschool age in their families.

There appears to be no difference in the degree of immunity between those individuals who have developed antitoxin from natural causes and those who did so because of the stimulus of the toxin-antitoxin injections.

Institutions in which the children have been given the immunizing injections have been remarkably free from diphtheria.

The schoolchildren who have been injected have had one-fourth as many cases as the untreated children, and these cases have been of less severity.

THE DEGREE OF IMMUNITY TO DIPHTHERIA INSURED BY A NEGATIVE SCHICK TEST

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WILLIAM H. PARK

This communication was suggested by reading an article on an outbreak of tonsillitis suspected to be diphtheritic by Dr. S. A. Blauner.[1] Through the courtesy of Dr. Blauner, I was enabled to study the outbreak described. I saw the last three cases in the acute stage, and made cultures from the sick and convalescent children and from many of the healthy children distributed in the different parts of the institution. The laboratory and clinical material considered together lead me to somewhat different conclusions from those drawn by Dr. Blauner. Although the results of all the laboratory examinations were sent to him, he either failed to remember them or did not interpret them as I do.

Before considering this special outbreak, it seems desirable to state the evidence which supports the view that sufficient antitoxin in the body to prevent the development of a positive Schick test under ordinary conditions will prevent the development of diphtheria. There can be no doubt that the injection of 500 units of antitoxin will give children an immunity for a period of at least twelve days and usually twenty days. The proof of this is derived from its use in many outbreaks and in the exposed children of hundreds of thousands of families. The recent statement by Haven's that about 20 per cent. of diphtheria bacilli are of a different type from the one used to produce toxin for the immunization of horses, and that the available monovalent antitoxin will not completely neutralize the toxin made by this other type, and that, therefore, diphtheria may develop from infection with this other type in children showing a negative Schick test to the standard toxin, has not been borne out by experience nor confirmed by us in experimental work. The evidence that antitoxin is the only substance in the body that can prevent the toxin acting is also so conclusive that we can assume that a negative Schick test means the presence of sufficient antitoxin to neutralize it. The debatable points are: is the minimum amount of antitoxin required to prevent the Schick test sufficient to prevent the development of diphtheria and, if not, is it sufficient to prevent a toxemia. Closely bound up with the answer to these questions is the degree of certainty that the general practitioner can assume that, as ordinarily carried out, a negative Schick test proves the presence of at least this minimum amount of antitoxin. It will be wise to dispose of the last query first.

A Schick test requires the injection intracutaneously of one-fiftieth of a lethal dose for a 250 gm. guinea-pig in 0.1 c.c. or an equivalent amount of toxin in a different amount of fluid. We recommend one-fortieth M. L. D. in 0.2 c.c. because we find the larger amount of fluid easier to give and, therefore, safer for the inexpert. Reports sent us some time ago of several series of cases with no positive Schick reactions caused us to obtain outfits from various manufacturers. Dr. Zingher found in a considerable number of instances that the available quantity of toxin in some of the outfits was much less than the required amounts. The children tested with these solutions gave negative Schick reactions when control tests made with standardized toxins gave positive ones. More recent tests of outfits from these firms have given uniformly good results.

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[1] Am. J. Dis. Child. 21:472 (May) 1921.

Not only must the toxin be measured accurately, but the toxin must be delivered wholly intracutaneously. If injected a little too deeply it will not be retained in the skin and will not exert the desired effect. If the small white area does not appear at the point of injection the Schick test cannot be relied upon. Careful investigation leads us to believe that from 2 to 4 per cent. of the persons giving negative Schick tests do so because of error in technic. When great care has been taken to have the toxin strength correct and the injection given strictly intracutaneously, we have not discovered any errors. Too strong a toxin will give a larger percentage of positives than will the standard amount. The endeavor of Schick was to choose the amount that would as nearly as possible divide the susceptible from the immune.

During the past seven years my colleagues in the laboratory have made over 150,000 Schick tests, and have immunized about forty thousand of those giving a positive reaction. We have also tested from year to year about fifteen thousand children who are inmates in institutions, so as to note any changes. There has been an average difference of about 2 per cent. in the annual readings. This we have considered as partly due to borderline cases and partly to errors in technic. The major part of this work has been directly carried out or has been supervised by Dr. A. Zingher and Dr. M. C. Schroder. We have during these years earnestly sought to discover all cases of suspected diphtheria which occurred among those who had given negative Schick reactions, either before or after the toxin-antitoxin injections.

During this time we have learned of and investigated eighteen cases of suspected diphtheria in those who had given a negative Schick reaction. Two of these were in an institution in Brooklyn, two in an institution in New York, eight in Dr. Blauner's institution and six in their homes. In none of these cases has death occurred. Undoubtedly, there have been other cases concerning which information has not reached us. We are now cataloging 100,000 New York City school children who have given negative Schick tests, and 100,000 untested children from the same schools.

During the next year we are going to look up every case reported as diphtheria and determine the difference between the incidence of diphtheria in the two groups of children. In as far as possible, we will inquire into each case so as to have both the physician's original report and the results of the inspector's investigation. We hope that, by this method, we may get a decision within a year as to the degree of immunity from diphtheria insured to children giving a negative Schick reaction.

It is very difficult to decide whether any of the sixteen cases with positive cultures, having patches of pseudomembrane either confined to the tonsils or spreading to the pharyngeal borders, were cases in which a faulty Schick test had been made, or whether they must be considered moderate cases of diphtheria developing in spite of an amount of antitoxin sufficient to prevent a positive Schick test, or whether they were due to other microbes, the diphtheria bacilli being present by accident and taking no active part. An important fact to remember is that, whatever the final decision is as to the etiology of these cases, they are extremely rare.

A detailed discussion of the outbreak that occurred in Dr. Blauner's service will show the complexity of the factors involved. We will combine a consideration of the clinical observations supplied by Dr. Blauner and the laboratory findings of Miss Mann who carried out extensive investigations under the general direction of Dr. A. W. Williams and myself.

The clinical facts can be stated briefly as follows: In a ward of about twenty children ranging from 2 to 5 years of age, all but one of whom had given a negative Schick reaction, three cases of croupous tonsillitis developed December 23, one on the twenty-sixth, two on the twenty-eighth, one on the twenty-ninth and two on the thirty-first. Eight of these showed grayish patches on the tonsils. In five, the patches spread from the tonsils to the edges of the phraynx. One of the two cases developing on the thirty-first was mild and had no exudate. The temperature of the cases ranged from 102 to 104 F. on the first day, and dropped rapidly by lysis.

Dr. Blauner states in his article that at first he had no suspicion that the earliest three cases had diphtheria because of the previous negative Schick reactions, but when the positive laboratory reports came in he became convinced that they were cases of true diphtheria. The three cases I saw would have been considered by me as probably, but not at all certainly, diphtheria. The cultures from all the cases, except the ninth, which was very mild, contained virulent diphtheria bacilli. The mild case contained no diphtheria bacilli.

Four of the eight moderately severe cases had given negative Schick reactions on their entrance into the institution; three had become negative after injections, and one, though injected, had remained positive. The first seven[3] of the children received antitoxin and recovered promptly; the last two received no antitoxin and recovered equally rapidly. The possibilities seemed to be (1), that the amount of antitoxin as shown by the negative Schick reaction had been in these cases insufficient to prevent moderate diphtheria; (2) that some other infection had caused the croupous tonsillitis, and that the diphtheria bacilli were accidentally present in carriers, and (3) that the sore throat started by the pyogenic organisms had paved the way for the local action of the accompanying diphtheria bacilli. To test out these possibilities we omitted giving antitoxin in the last two cases which had just developed and one of which was very mild. These two cases progressed favorably as I felt certain they would and the children recovered as promptly as did those that had received antitoxin. This result suggested that whether or not the diphtheria bacilli had taken part in the formation of the exudate, the recovery was probably not due to the additional antitoxin injected.

A week later, January 7, cultures were made from twenty-seven of the children in the room in which the infected cases occurred, and from eleven of them virulent diphtheria bacilli were obtained. As these children had been given an immunizing injection of antitoxin, Dr. Blauner thought that the cessation of the cases, in spite of the presence of so many carriers, was a strong point in favor of the former amount of antitoxin having been insufficient to protect. This led me to make cultures from nineteen children taken at random from other parts of the building where no antitoxin immunization had been given. To our surprise, we found the carriers even more abundant here than in the ward from which the cases came. Miss Mann isolated diphtheria bacilli from the cultures made from ten of nineteen children (See accompanying table). All strains were tested and found to be toxin producers. The children in the general building were, therefore, apparently protected by the amount of antitoxin which had given a negative Schick reaction. With these results before us, we see that if the tonsillitis in the ward had been caused by some other infection than diphtheria, the throats, if not already infected, would almost necessarily have become carriers of the bacilli. Dr. Blauner's complete dependence on the culture results to solve the nature of the infection is not surprising, since health departments have so emphasized the value of the bacteriological reports in suspected cases of diphtheria that many physicians believe that a positive report from a suspected case proves that the case is one of diphtheria.

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[3] There is some confusion as to whether it was the eighth case or an additional case that received no antitoxin. There is no doubt that there was a case with tonsillar exudate similar to the eighth case.

The absence of diphtheria bacilli in a culture properly made is fairly good proof that no such bacilli are in the throat and, that, therefore, the case is not one of diphtheria. If diphtheria bacilli are present, the probability of diphtheria being present is greater or less according to the more or less characteristic appearance of the lesions. The laboratory indicates the presence or absence of the bacilli and the danger of the case spreading infection; the clinical examination determines the probability or certainty of the presence of diphtheria. It would be more helpful if the laboratory could make the diagnosis, but, unfortunately, it cannot. If we apply the above facts to Dr. Blauner's cases, we see that, as certainly half of the children in the institution were carrying virulent diphtheria bacilli in their throats during the month of January, whatever nasal or throat infection took place diphtheria bacilli would probably be associated with it. With 50 per cent. of the normal cases carrying bacilli, practically every diseased child would almost immediately become a carrier.

RESULTS OF THE CULTURE EXAMINATION OF NINETEEN HEALTHY CHILDREN FROM WARDS IN WHICH NO DIPHTHERIA HAD OCCURRED

Name	Result	Name	Result
A. R.	Negative	L. E.	Diphtheria bacilli
A. M.	Diphtheria bacilli	L. M.	Diphtheria bacilli
B. F.	Diphtheria bacilli	M. L.	Diphtheria bacilli
B. A.	Diphtheria bacilli	R. L.	Negative
B. W.	Negative	S. L.	Negative
D. M.	Diphtheria bacilli	S. S.	Diphtheria bacilli
G. I.	Negative	W. S.	Negative
H. M.	Negative	W. J.	Diphtheria bacilli
H. D.	Diphtheria bacilli	Z. J.	Negative
H. M.	Negative		

CONCLUSIONS

A negative Schick test in cases which have an active immunity, either natural or acquired, when the toxin used and technic employed have been suitable indicates an almost complete security from diphtheritic disease, not only for the immediate time but for the future.

Those that have natural antitoxin and those who acquire it through toxin-antitoxin injections may harbor diphtheria bacilli, and if they later suffer from tonsillitis, due to other microbes, throat cultures will contain diphtheria bacilli. The positive culture alone suggests but does not establish that the suspected case is one of diphtheria.

Under unusual conditions it is possible that, when diphtheria bacilli are present in a throat which becomes the seat of some other infection, they may develop their toxin and cause superficial lesions in the mucous membrane even though the cases have sufficient antitoxin to give a negative Schick reaction. Cases which present this possibility are rare, and when met with by us have done well without additional injection of antitoxin.

In the outbreak discussed in this communication, the fact that, while an extraordinarily large percentage of all the children in the institution were carriers of virulent bacilli only one group develop cases of tonsillitis with pseudomembranous patches, makes it uncertain whether the cases were diphtheria.

THE DURATION OF THE IMMUNITY CONFERRED BY THE USE OF DIPHTHERIA TOXIN-ANTITOXIN

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By M. C. Schroeder

The practical value of the toxin-antitoxin immunizing injections depends on several factors: the harmlessness of the injections, the proportion of the susceptibles who respond and become immune and finally the duration of the immunity. Active immunization would have been attempted on a large scale years ago except for the difficulty of identifying those who required protection.

For twenty-five years it has been known that it was possible to immunize with the toxin-antitoxin. In fact in our own laboratory we learned this in 1896 and used the method practically in horses. The suggestion of Schick that the introduction of a minute quantity of toxin in the skin would differentiate those that had antitoxin immunity from those that had none, made it possible to inject those who had no antitoxin and watch the results. This led Behring to undertake active immunization in Germany nine or ten years ago. His success encouraged us to undertake the same work in New York. After we had determined the harmlessness of the mixture and the percentage of those becoming immune after one, two and three injections (see table), we realized that the all-important fact which remained to be discovered was the duration of the immunity. Even if we could make every child immune this would be of little practical value unless the condition remained permanent. We decided that the most favorable means of determining the truth as to this point was to seek institutions where the children were held for a number of years. Dr. Zingher and the writer picked out a number of institutions and we have watched the children from year to year. Five and one-half years have elapsed since the children in the first institution were treated. It is difficult to wait with patience for the years to pass to reach the final conclusion, but there is no other way. We have also tested over 4,000 inmates of the State Institution for the Insane in order to obtain results on a group of people likely to remain located indefinitely, whereas the children in institutions often make but a short stay and the majority will have left in a few years.

Table I gives the test results in 28 children that have remained in one institution for five years.

It is seen that when once antitoxin develops in those injected with toxin-antitoxin it is quite or almost as persistent as in those who develop it naturally. It lasts in the great majority for at least five years.

Response to Toxin-Antitoxin Stimulation in Infants. Over 2,400 infants ranging in age from a few hours to two weeks were tested and immunized by Miss Denny, Miss Alper and the writer. When re-tested one year later we found that only 50 per cent. had become immune. This showed that active immunity is not readily produced in these young infants by toxin-antitoxin injections owing partly to the inhibitive effect of the passive immunity derived from the mother. This passive immunity is lost later. It is found that toxin-antitoxin can only be depended upon to act effectually after the age of six months.

TABLE I.

SHOWING RESULTS OF 28 CASES DURING 5½ YEARS RE-TEST PERIOD

	Schick Test	Toxin-Antitoxin—3 injections					Schick Re-tests during 5 years					
	Nov. 16, 1915	Nov. 20 & 25,		Dec. 3, 1915	March 20, 1916	May 19, 1916	June 29, 1916	Nov. 9, 1916	July 22, 1917	Jan. 13, 1919	Jan. 11, 1921	Jan. 1922
Case 1	++	+	+	+	—	—	—	—	—	—	—	—
2	+	+	+	+	—	—	—	—	—	—	—	—
3	+	+	+		—	—	—	—	—	—	—	—
4	±	+	+		±	±	—	—	+ =	—	—	—
5	+	+	+	+	—	—	—	—	—	—	—	—
6	++	+	+	+	+ =	—	—	—	—	—	—	—
7	±	+	+	+	—	—	—	—	—	—	—	—
8	±	+	+		—	—	—	—	—	—	—	—
9	++	+	+	+	—	—	—	—	—	—	—	—
10	+	+	+	+	—	—				—	—	These children were lost track of.
11	±	+	+		—	—	—	—	—	—	—	
12	+	+	+	+	—	—	—	—	—	—	—	
13	+	+	+	+	—	—	+ =	—	—	+	—	
14	++	+	+	+	—	—	—	—		—	—	
15	+	+	+	+	—	—	—	—	—	—	—	
16	+	+	+	+	—	+ =	—	—	—	—	—	
17	+	+	+	+	—	—	—	—	—	—	—	
18	+	+	+	+	±	—	—	—	—	—	—	
19	+	+	+	+	+ =	—	—	—	—	—	—	
20	±	+	+		+ =	±	—	+ =	+ =	±	—	
21	+	+	+	+	±	—	—	—	—	—	—	
22	±	+	+	+	—	—	—	—	—	—	—	
23	+	+	+	+	—	—	—	—	—	—	—	
24	+	+	+	+	—	—	—	—	—	—	—	
25	++	+	+	+	—	—	—	—	—	—	—	
26	±	+	+		—	—	—	—	—	—	—	
27	+	+	+	+	+	—	—	+	+ =	—	—	
28	+	+	+	+	—	—	—	—	—	—	—	
					4± 3+ = 21—	2± 1+ = 25—	1+ = 27—	1+ 1+ = 26—	3+ = 25—	2+ 26—	28—	

*Thirteen received 2 injections 1½ c.c. 1-27-19.
*Twenty received 2 injections 1½ c.c. 1-27-19.
— = negative.
+ = positive.

Twenty-one of 28 became negative within 4 months and remained negative 5 years.
Twenty-five of 28 became negative within 6 months and remained negative 4½ years.
The remaining 3 became — but relapsed more or less; but became permanently — after two additional injections.
Only nine remained to 1922. These were all negative.

TABLE II.

	Total Tested	Doses T A T	Months After	Shick Results		Re-test Percentage Becoming Immune
				11—		
St. Joseph's	12	3	60	*1+		100
Ages mostly	38	2 or 3	*38	38—		100
				Boys	Girls	
				64—		
5—10 years	78		10	9+	5+	94
				*91—		
	120	2 or 3	3	11+	18+	85
	8	1 or 2				
St. Agnes	3	2	60	3—		100
3—14 years	25	2	20	25—		100
				Girls	Boys	
	54	2	12	50—	4+	92
	38	2 or 3	38	33—	2+	94.8
St. Dominick				6—	1—	
Ages mostly	10	1	31	1+	2+	70
9—12 years				14—		
	16	2	18	1+	1+	93.8
St. Malachy's				13—		
Ages (5—15 yrs.)	14	2	14	1—		100
Randall's Island				42—	4—	89.2
(5—30 years)	49	2	33	2+	1+	94.9
Hebrew Orphan	68	3	32	59—	3—	92.6
Asylum (5—15 yrs.)				4+	2+	
Mt. Loretto	83	1 or 2	3	62—	12—	
				3+	7+	77.5
House of Refuge	7	1 or 3	35	4—	2—	71.5
(10—20 yrs.)				1+		
Leake & Watts	32	3	27	26—	2—	93.8
(5—15 yrs.)				3+		
					1+	
	23	2 or 3	24	19—	3+	82.6
N. Y. Catholic	19	3	6	17—	2+	89
Protectory				1+		
(4—10 yrs.)	20	3	12	18—	1+	95
	55		22	53—	2+	96.4
N. Y. Foundling	32	3	17	30—		96.9
(1—5 yrs.)				1+	1+	
Chldrns. Stations	350	3	7	314—	37+	89
3 mos. to 4 yrs.						

*1 case became positive, but when reinjected became negative.

The results upon re-test were as follows:

Group	No. of Cases	Reaction Changed From Positive to Negative	Reaction Remaining Positive	Percentage Becoming Immune
A	105	85	20	80%
B	245	209	36	85%

Response in School Children. Another side of the problem was presented by the use of toxin-antitoxin among the public school population. During the past two years fully 50,000 children attending the public schools of Brooklyn have been Schick tested and immunized under my personal supervision.

During the past month we have begun re-testing those children who were immunized.

The results are given in Table III:

School	No. of Children Retested	No. of Children Becoming Negative	No. of Children Remaining Positive	Percentage Becoming Immune
P.S. 16, Bklyn.	288	260	28	90%
P.S. 142, Bklyn.	157	148	9	94%
P.S. 50, Bklyn.	125	119	6	95%

The above children received three doses of 1 c.c. each. In P. S. 149, Brooklyn, 35 children received two doses of 1½ c.c. each. Of these children, 26 became negative, 9 remained positive and were re-injected. The latter will be tested again later.

Acquired Immunity in Adults. During the past year 4,396 inmates of Kings Park State Hospital were Schick tested and 555 patients were immunized. These patients were divided into two groups, Group A receiving one inoculation of toxin-antitoxin and Group B, two inoculations in 1½ c.c. doses.

Such, in brief, is a résumé of part of the work done during the past 5 years to test out the possibilities of the duration of conferred immunity by means of diphtheria toxin and antitoxin mixtures.

For bibliography see the original article.

ACCURACY OF THE SCHICK REACTION

Influence of Variations in Diphtheria Toxin Content in Schick Outfits

ABRAHAM ZINGHER

The rapid increase in the popularity of the Schick reaction as a method for determining the susceptibility or immunity of individuals to diphtheria is based on two factors: (1) the great reliability of the test, and (2) the ease with which it can be carried out.

Dr. Park and I have repeatedly emphasized the need of observing with care three points that are absolutely essential if the test is to be reliable. They are: (*a*) a standard toxin dilution of proper strength; (*b*) accurate technic in the injection by means of a good syringe and a suitable fine needle, and (*c*) an accurate interpretation of the reaction.

RELIABILITY OF THE SCHICK TEST

Accumulating experience and reports from many sources, and our own experience at the Willard Parker Hospital and in various other institutions during a period of seven years, have established the Schick reaction as a most valuable clinical test for determining immunity from diphtheria or susceptibility to the disease. Most striking, indeed, have been the results noted at the Willard Parker Hospital. Of several thousand scarlet fever patients, more than 2,200 had given a negative Schick reaction on admission to the institution. No prophylactic injections of antitoxin were given to these patients, as they were considered to be immune to diphtheria. They were carefully observed and, it is interesting to note, not one of these patients developed undoubted clinical diphtheria even though they were subsequently exposed to cases of diphtheria developing among some of the Schick-positive patients; indeed, many of them had become temporary carriers of virulent diphtheria bacilli during their stay in the hospital.

The great reliability of the test is further emphasized by our recent experience in several institutions in which outbreaks of diphtheria had occurred. The Schick test was made on all the children of the institutions, and only the positive reactors were passively immunized with antitoxin. In each institution the outbreak was thus promptly controlled, and no cases of diphtheria developed among the Schick-negative children, although they had received no immunization with antitoxin.

It is important from a practical standpoint to remember that individuals who are carriers of virulent or nonvirulent diphtheria bacilli may develop a septic sore throat due to the streptococcus or some other organism. Such carriers as a rule give negative Schick reactions and are therefore immune to diphtheria. The presence of a lesion resembling a diphtheritic exudate in these individuals, associated with a positive throat culture, would be sufficient evidence to many observers that the lesion was diphtheria. In several such patients, however, in whom we were certain that the negative Schick reaction was correct, antitoxin was not injected, although we were prepared to give it if the symptoms became worse. Repetition of the Schick test again gave negative results. Specimens of blood were taken from these patients and their antitoxin content determined. All these patients had from 1/10 to 2 units of antitoxin per cubic centimeter of blood serum which, according to von Behring, would be from ten to 200 times as much as was necessary to protect the individuals from diphtheria. Antitoxin, however, should always be given when the suspicion of the presence of diphtheria is strong, because there is always the possibility that there has been an error in the technic of the test or in its reading, and we are not justified in taking unnecessary risks. Failure of improvement in such a case after the administration of antitoxin will generally show that we are dealing with a nondiphtheritic sore throat in a carrier of diphtheria bacilli.

Sources of Possible Error Connected With the Schick Test

I have been informed by various observers of cases in which patients giving negative Schick reactions subsequently developed diphtheria. I firmly believe that in such cases one or more of the three factors enumerated above, i. e., the strength of the toxin, the technic of the injection, or the interpretation of the reaction, had been at fault. In several instances we were able to show that the technic itself was inaccurate. Errors in the reading or the interpretation of the test accounted for some of the poor results. At times a Schick reaction was read as negative after a superficial observation at the end of twenty-four or forty-eight hours, when a later reading would have disclosed a positive reaction. This was indicated by the definite pigmentation remaining at the site of the test and seen at a subsequent examination. Such pigmentation should have been looked for and would probably have been found in some of the cases that were reported as clinical diphtheria developing in Schick-negative children. To interpret and identify the pseudoreaction, moreover, it is necessary to make a control test with the heated toxin as I have recommended. When a large number of very young children are to be tested, the control could often be omitted for the sake of economy in time, in view of the relative infrequency of the pseudoreaction in this age group.

The first essential condition for a reliable Schick test is to have a standard toxin dilution of proper strength. It is possible for the individual observer to guard against faulty technic and inaccurate reading of the reaction, but he is forced to depend on the outfits supplied by the various laboratories for a suitable toxin preparation. It is important to realize that individuals who give a strongly positive Schick reaction with standard dilutions of toxin may give only a faintly positive or *a negative* reaction with weaker dilutions.

Table 1 illustrates the effect of three different dilutions of toxin on groups of children who had given strongly positive, moderately positive and faintly positive reactions, respectively, with a standard dilution. Variations

TABLE 1.
EFFECT OF THREE DILUTIONS OF TOXIN*

Dilutions of Diphtheria Toxin	Groups of Children 1	 2	 3
1. Standard dilution, 0.2 c.c. = 1/50 M. L. D.....	+	±	$\underline{\pm}$
2. One-half standard dilution, 0.2 c.c. = 1/100 M. L. D.	±	$\underline{\pm}$ or —	—
3. One-quarter standard dilution, 0.2 c.c. = 1/200 M. L. D.	$\underline{\pm}$ or —	—	—

*The signs used in this and the following tables should be read thus: + strongly positive Schick reaction; ±, moderately positive Schick reaction; $\underline{\pm}$, faintly positive Schick reaction; —, negative Schick reaction.

in the reactions shown by the same individuals to apparently the same dilutions of toxin can thus be explained by differences in the strength of the dilutions used in the test.

My attention was recently drawn to the possibility that some of the outfits supplied by commercial laboratories were below standard strength. A private physician reported that he had used a Schick outfit from a commercial laboratory on three children in one family and had obtained negative reactions in all the children. He suspected that something was wrong with the toxin dilution, and retested the three children with an outfit from the Research Laboratory. Two of the children now gave positive reactions and one a negative reaction. The importance of this observation prompted me to ask several commercial laboratories for a number of Schick outfits, which they manufacture for public sale, and to test them. These outfits were secured directly from the manufacturers so as to avoid the possibility that the outfits had deteriorated while in the hands of the retailer. A series of children who had given positive Schick reactions with the outfits furnished by the Research Laboratory was used for the tests. All the outfits from one laboratory were tested simultaneously on the same children, and at the same time a control test was made with one of the outfits of the Research Laboratory.

The results noted were very interesting and are of considerable importance in showing one of the great possible sources of error in the results of the Schick test.

RESULTS OBTAINED WITH COMMERCIAL LABORATORY OUTFITS

In reading Table 2 and the subsequent tables the vertical columns indicate the different reactions given by the various outfits on the same child, and the horizontal columns the reactions shown by one outfit on various children.

Table 2 shows that Outfits 1 and 2 were inactive and gave negative reactions in all children. It is important to note that not even a faintly positive reaction was noted in those children who had shown a strongly positive reaction with the control test. Outfit 3 gave reactions about twice as strong as those noted with the control test, while Outfits 4 and 5 gave reactions similar to those shown with the control test.

TABLE 2.

RESULTS WITH OUTFITS OF COMMERCIAL LABORATORY I.

	Children Tested						
Schick Outfits	A	B	C	D	E	F	G
Control (Research Lab.)	+	+	+	±	$\underline{\pm}$	±	+
1	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—
3	++	+	++	+	±	+	++
4	+	+	+	±	$\underline{\pm}$	+	+
5	+	+	Not tested	+	$\underline{\pm}$	+	Not tested

Table 3 shows that Outfit 1 gave a moderately positive reaction in one child showing a strongly positive reaction with the control test, and faintly positive reactions in two children giving moderately positive reactions with the control outfit.

TABLE 3.

RESULTS WITH OUTFITS OF COMMERCIAL LABORATORY 2

	Children Tested		
Schick Outfits	A	B	C
Control (Research Lab.)	+	±	±
1	+	$\underline{\pm}$	$\underline{\pm}$
2	±	$\underline{\pm}$	$\underline{\pm}$
3	$\underline{\pm}$	—	—

Outfit 2 gave a faintly positive reaction in the child showing a strongly positive reaction with the control test, and very faintly positive reactions in the two children giving moderately positive reactions with the control outfit.

Outfit 3 gave a very faintly positive reaction in the child showing a strongly positive reaction with the control test, and negative reactions in the two children giving moderately positive reactions with the control test.

The directions for the tests given in the circulars of the outfits of Laboratory 3 state that 0.1 c.c. should be injected. We injected 0.2 c.c. so as to make the area of the reaction conform to that of the control test, in which 0.2 c.c. is used. In Child E (Table 4), Outfits 1 and 2 gave negative reactions when 0.1 c.c. was injected, but faintly positive reactions when 0.2 c.c. was injected.

TABLE 4.

RESULTS WITH OUTFITS OF COMMERCIAL LABORATORY 3

	Children Tested				
Schick Outfits	A	B	C	D	E
Control (Research Lab.)	+	+	+	+	±
1	+	+	+	+	$\underline{\pm}$
2	+	+	+	+	$\underline{\pm}$
3	+	+	+	+	±
4	±	±	±	+	$\underline{\pm}$

Table 4 shows that three of the outfits gave reactions similar to the control test in four children; the fifth child showed a faintly positive reaction with Outfits 1 and 2. Outfit 4 gave moderately positive reactions in three of the children who had strongly positive reaction with the control outfit. The capillaries containing the toxin in these outfits were of fairly thick glass, and one experienced considerable difficulty in breaking them to make the necessary dilution. The clinical results with the outfits of this laboratory corresponded quite closely to those noted with the control outfit. It must be remembered, however, that we used twice the amount of diluted toxin directed by the circular in these outfits, and injected 0.2 c.c. instead of 0.1 c.c. in making the tests.

The contents of one capillary were also injected into a guinea-pig weighing 250 gm. Each capillary was supposed to contain one minimal lethal dose of toxin; therefore the animal should have died on the fourth day after receiving the injection. The guinea-pig showed local induration, lost in weight and became quite ill, but ultimately recovered. This result showed that the capillary contained less than one minimal lethal dose of toxin, the amount required to make the standard dilution for that outfit.

Table 5 shows that the outfits of this laboratory gave results which corresponded quite closely to those noted with the control outfit. Outfit 5 was perhaps slightly stronger than the control.

TABLE 5

RESULTS WITH OUTFITS OF COMMERCIAL LABORATORY 4

	Children Tested			
Schick Outfits	A	B	C	D
Control (Research Lab.)	+	±	±	+
1	+	±	+	+
2	+	±	±	+
3	+	±	±	+
4	+	±	±	+
5	+	±	+	+

RESULTS OBTAINED WITH RESEARCH LABORATORY OUTFITS

In order to determine how uniform the outfits of the Research Laboratory are, several of them were tested in the same manner as the outfits supplied by the commercial laboratories.

This outfit is prepared at the Research Laboratory with a stable ripe toxin, of which the minimal lethal dose is 1/70 c.c. The capillary tubes of the outfits are filled by means of a 0.25 c.c. original record syringe (the Kny-Scheerer Company) the barrel of which is divided into fifteen equal parts. One subdivision of the syringe corresponds to 1/60 c.c., and this is the amount of toxin filled into each capillary tube. The contents of the capillary tube represent, therefore, a little more than one minimal lethal dose of toxin, the slight excess (about 14 per cent.) allowing for some deterioration of the toxin. When the toxin is diluted with the 10 c.c. of saline contained in the outfit, the solution contains 1/50 minimal lethal dose in 0.2 c.c., the amount recommended for the test. Allowing for wastage of some of the diluted toxin, one outfit will be sufficient for at least thirty-five Schick tests.

Table 6 shows that the first four outfits gave quite uniform results. Outfit 5 gave a moderately positive reaction in Children A and C, who had shown strongly positive reactions with the other outfits. A slightly deeper injection may account for these reactions.

TABLE 6.

RESULTS WITH RESEARCH LABORATORY OUTFITS

	Children Tested				
Research Lab. Schick Outfits	A	B	C	D	E
1	+	+	+	±	⩲
2	+	+	+	±	⩲
3	+	+	+	±	⩲
4	+	+	+	±	⩲
5	±	+	±	±	⩲

Three guinea-pigs, weighing 250 gm., were each injected with the contents of one of the Research Laboratory outfits One animal died of acute diphtheria intoxication between the third and fourth day, and the other two died between the fourth and fifth day.

The results noted with these outfits show a considerable degree of uniformity in the clinical tests. The animal tests also show that each of the outfits contained one unit of toxin, the amount required for a proper standard dilution.

It is evident, therefore, that it is possible to manufacture outfits for the Schick test on a large scale without sacrificing accuracy.

Summary and Conclusions

1. The accuracy of the Schick test depends on the strength of the toxin, the technic of the test and the interpretation of the reaction.

2. Errors in any of these may make the results of the test unreliable. It is possible to guard against a poor technic and a wrong interpretation of the reaction, but it is essential to be able to depend on the accuracy of the outfits for the Schick test obtainable on the market.

3. The outfits furnished by four commercial laboratories were tested. Of five outfits of one laboratory, two contained too small an amount of toxin to give a positive reaction in Schick-positive children. Of three outfits of the second laboratory, one was somewhat weaker and two very much weaker than the standard outfit, so that negative reactions were obtained in Schick-positive individuals. The outfits of the third laboratory compared fairly well with the control in the clinical test when twice the amount of toxin dilution prescribed in the directions on the outfits was injected. The animal test showed only about 85 or 90 per cent. of a minimal lethal dose in an outfit instead of one minimal lethal dose. The outfits of the fourth laboratory gave reactions similar to those noted with the control test.

4. The outfits furnished by the Research Laboratory gave practically uniform results clinically, and the animal test showed full potency of the toxin.

5. It is practicable to manufacture outfits for the Schick test on a large scale without sacrificing accuracy.

DIPHTHERIA PREVENTIVE WORK IN THE PUBLIC SCHOOLS OF NEW YORK CITY

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By Abraham Zingher

1. *Introduction.* The very successful results obtained by us and by other workers in the active immunization of large numbers of children with diphtheria toxin-antitoxin has stimulated the Department of Health of New York City to begin an active campaign in diphtheria preventive work in the public and parochial schools of this city. Permission was obtained from the Department of Education to carry out the Schick test and the necessary active immunization in about 250 public schools. Beginning in 1916. a number of schools were Schick tested in the Boroughs of Manhattan, the Bronx and Brooklyn. During the period of the war the work was limited in extent on account of lack of necessary personnel.

Through the coöperation of the Manhattan Chapter of the American Red Cross, Dr. Park obtained the financial aid necessary to start this work on a more extensive scale. A number of physicians, nurses and assistants were employed and divided into groups. In Brooklyn and Queens the work was done under the direction of Dr. Schroeder while in Manhattan and the Bronx, 2 groups have been working under direction of the writer. Between the end of February, 1921, and the end of this school year, we have applied the Schick test in 44 of the larger schools in these 2 boroughs and have tested over 52,000 children in Manhattan and the Bronx. Those children who gave a positive or a positive combined reaction were injected with toxin-antitoxin. In the first 22 schools, the injected children were re-tested with the Schick test before the end of the school year, in order to determine which children had developed an active immunity against diphtheria. The following was the method followed in the schools.

2. *Introductory Procedure Before Schick Testing a School.* An interview was first obtained with the principal or assistant principal, the Schick test and toxin-antitoxin immunization were explained, and the coöperation of the school authorities was invited. Consent blanks and circulars of information to parents were left at the school to be distributed to all the children. Conference meetings were arranged at which the teachers were addressed by the principal or a physician, and their coöperation also requested. Each child was required to return the consent blank signed by the parent, with an affirmative or negative statement. The teachers later made out alphabetical lists of the names of those children whose parents had given their consent. Special sheets, called class sheets, were used for this purpose and also for recording subsequently the results of the Schick test and the injections of toxin-antitoxin.

Procedure on the Day of the Schick Test. On the day of the test, the children are brought to a class room where the physicians and nurses are doing the work. By good coöperation on the part of the principal and teachers, we are able to apply the Schick Test and the control test to as many as 500 or 600 children per hour. In one school we tested on one day, during school hours, 2400 children; in another double school, over 2700 children. The needles are thoroughly cleaned and sterilized between the tests by wiping them off with a cotton sponge which has been placed in a Petri dish and saturated with 95 per cent. ethyl alcohol. A 1.0 c.c. "Original Record" syringe and a 26 gauge ¼ inch steel needle were found very serviceable for the test.

4. *The Dilutions for the Schick Test and for the Control Test.* The Schick outfits[1], so generally used at the present time and so well adapted to the work where small numbers are to be tested, are not very suitable for the testing of large numbers of children. The dilutions for our work are therefore prepared from bulk toxin, as described in a previous communication[2]. The dilutions are made according to the M.L.D. strength of the toxin. Our toxin had an M.L.D. of 0.01 c.c. A dilution of 1.0 c.c. of toxin in 1,000 c.c. of saline gives a strength of toxin of which 0.2 c.c.=1/50 M.L.D. To make the reactions more distinct, we add 25 per cent. excess of toxin and dilute 1.25 c.c. of toxin in 1,000 c.c. of saline. The dilution thus prepared represents 1/40 M.L.D. in each 0.2 c.c.

For the control test dilution. Instead of 1.25 c.c., we use 1.5 c.c. of toxin, heated to 75°C. for ten minutes and diluted with 1,000 c.c. of saline. The 20 per cent. excess of heated toxin in the control dilution as compared with the unheated toxin used in the Schick dilution was added for the purpose of allowing for any deterioration that might have taken place in the bacillus protein during the process of heating. By observing this precaution we found that children showing a negative pseudo-reaction would have an area of redness and later pigmentation equal in size and appearance on both forearms. In consequence there was less danger of mistaking a negative pseudo-reaction for a combined positive reaction.

The dilutions were freshly prepared each day for the day's work. No diluted toxin was kept over from one day to another.

The rule was adopted always to make the Schick test on the right forearm about 2 to 3 inches below the bend of the elbow and the control test on the left forearm in a corresponding location. One knew then exactly where to look for the reactions and could thus avoid confusion in the readings.

Procedure on the Day of the Readings of the Schick Reaction and of the First Injection of Toxin-Antitoxin. The reactions were read from 3 to 7 days after the Schick tests. By reading the tests before three days have elapsed the interpretation of the reactions is not as accurate. Readings made on the third and fourth days showed the fading pseudo-reactions somewhat better, but a few doubtful reactions still remained, especially of the positive combined type. These were more accurately interpreted at the later readings. Very faintly positive Schick reactions, however, were likely to show no redness and only a slight brownish pigmentation when they were seen on the seventh day.

I. *Results of Schick Test.* The results with the Schick test in 44 schools are interesting and significant. They show that:

1. Children from the homes of the more well to do have a much higher percentage of positive Schick reactions than those from the homes of the poorer classes of the population, who live in closely crowded neighborhoods. The table shows that in some schools as many as 67.0 per cent. of the children were found to give the positive reaction. The percentage diminishes as we follow the schools down on the table until we reach schools located in the densely congested sections of the East Side, where not more than 16 to 20 per cent, gave positive reactions.

2. *Contact immunity* would seem to be an important element in the establishment of the so-called "natural immunity." Repeated exposure to the diphtheria bacillus in the congested districts not only causes actual clinical cases of diphtheria to develop but also probably produces mild infections

of the mucous membranes which are not recognized as diphtheria, but which may lead to the gradual development of an antitoxic immunity. This theory has received striking support from the results of the Schick testing of two private schools and one rural school*. At the Lawrenceville School near Trenton, N. J., 79 per cent. of boys, varying in age from 12 to 21 years, gave positive reactions; at the George School, Georgetown, Pa., 75 per cent. of the children gave positive reactions. In a rural school at Shilo, Cumberland Co., N. J., Dr. Knight of the New Jersey State Department of Health found that 85 per cent. of the children gave positive Schick reactions. These are striking figures and indicate that segregation of the children, either among the well to do or in rural and sparsely settled sections, probably plays an important part in retarding the development of natural immunity to diphtheria.

3. A study of the racial types, as represented by the child population of the different schools, gives other interesting data and shows that the *racial factor* must also be considered as one of the influences in the development of natural immunity.

(a) Children of the colored race, living often in congested neighborhoods, showed in spite of the crowded conditions a high proportion of positive Schick reactions. I had had occasion to note this racial peculiarity seven years ago at the Howard Colored Orphan Asylum at Kings Park, L. I., and a year later at the Colored Orphan Asylum at Riverdale-on-the-Hudson.

(b) Children of Italian extraction, living in the crowded East Harlem section of New York City, gave the lowest percentage of positive reactions.

(c) Children of Bohemian and Irish extraction gave about one-third positive Schick reactions.

(d) Children of Hebrew parents varied considerably in the percentage of positive Schick reactions. Those living in the congested sections of the City showed a low percentage, while those living in the upper West Side section of the City were among those who gave the highest percentage of positive reactions.

4. The *family factor* is also of importance in the development of natural immunity. This was brought out in some of our earliest observations on the results of the Schick test in groups of children belonging to the same family. We found that there was a marked tendency for all the children of the same family to show a similar Schick reaction, whether it was positive or negative. Where variations were found in the reaction, the younger children as a rule gave the positive, the older chlidren, the negative Schick reactions. The reverse condition, where older children gave a positive and younger children a negative reaction was very rare, except in families with young infants, who often gave a temporary negative reaction due to maternal immunity. These observations have been repeatedly confirmed in our more recent work in the public schools. While as a rule the living conditions and consequent exposure to diphtheria infection are very similar for all the children of the same family, yet we have seen that different families living under closely parallel conditions often show entirely different Schick reactions. With regard to antitoxin production there is probably an hereditary tendency on the part of the children of one family to respond either readily or slowly and poorly to repeated mild infections with the diphtheria bacillus.

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*I am indebted to Mr. Bowen, Secretary of the New Jersey State Department of Health for the results of the Schick test at the Lawrenceville School, and to Dr. David F. Weeks, Superintendent of the State Hospital for Epileptics at Skillman, N. J., for the results at George School.

This organism is as a rule universally prevalent in larger communities. For example, in New York City we always have on an average about 4 to 5 per cent. of bacillus carriers.

5. Natural antitoxic immunity in human beings can be interpreted therefore as due to a combination of factors in which *contact immunization* seems to play the most important part. The *racial* and *hereditary family factors* however, must also be considered as important elements in the development of such natural immunity.

6. The results show a fairly high proportion of negative pseudo-reactions among children of school age. It is interesting to note that there is a much higher proportion of negative pseudo-reactors among the immunes than there is of positive combined reactors among the non-immunes. We can therefore assume that repeated exposure to infection with the diphtheria bacillus brings about not only an antitoxic immunity, but also a hypersensitiveness of certain predisposed individuals to the diphtheria bacillus protein. The development of this hypersensitiveness renders the cells of the epidermis of the pseudo-reactor anaphylactic to the autolyzed protein of the diphtheria bacillus which is present in the test solution of toxin. The infrequent exposure of positive reactors to infection with the diphtheria bacillus accounts for the smaller number of positive combined reactions among them.

Among the colored children we noted a relatively low percentage of pseudo-reactions. There seems to be a racial tendency in these children to respond poorly to the antigenic action not only of the diphtheria toxin, but also of the bacillus protein.

II. *Results with Toxin-Antitoxin Immunization.* Table 1 shows results in a few of the schools in which the children receiving the toxin-antitoxin injections were re-tested. P. S. 90 was the only school in which the children had received *three* injections of toxin-antitoxin and the re-test was made after *five months.* In this school 87.5 per cent. of the injected children were found to have become immune as shown by a negative Schick reaction. The other injected children gave reactions that were still positive, but were very much fainter than the original reactions.

In the other re-tested schools, only two injections of toxin-antitoxin had been given from two to two and a half months previously. Two of these schools (P. S. 101 and 57), located in the more densely crowded sections of the City, showed 76.1 and 64.5 per cent. positive Schick reactions respectively. These results compare favorably with those noted in the other schools given in the table, in three of which we had used the same preparation of toxin-antitoxin (P. S. 43 Manhattan; 186 and 132). In the last two schools, however, the Schick re-tests were made after two months. In the remaining two schools noted in the table (P. S. 43 Bronx, and 46), a different mixture of toxin-antitoxin was used, but the results were about the same.

One feature of the Schick re-tests was noticeable. Most of the children who continued to give the positive reaction showed a much fainter area of redness than in the original test. In many instances the brownish pigmentation of the original positive reaction was still visible and was even at this time much larger in diameter than the area of redness of the re-test. The fainter reactions in the re-tests stood out also in striking contrast to the strongly positive reactions of children who have received the Schick tests for the first time.

Children who showed a positive reaction, even though it was very small in size and faint in appearance, received one or two additional injections of toxin-antitoxin. Schick re-tests will be made again next fall and we expect

TABLE 1

RESULTS OF RE-TESTS AFTER DIPHTHERIA TOXIN-ANTITOXIN IMMUNIZATION IN NEW YORK CITY PUBLIC SCHOOLS

P.S.	Location	Time Since Injection	Preparation Used	Doses Given	Amount	Total Children Retested	Schick Pos.	Schick Neg.	Per Cent. Neg.
90	148th St. & 8th Avenue	5 mo.	No. 24	3	1 c.c.	160	20	148	87.5
101	111th St. & Lexington Avenue	2.5 mo.	No. 23	2	1.5c.c.	152	31	121	76.1
57	115th St. & 3rd Avenue	2.5 mo.	No. 23	2	1.5c.c.	254	90	164	64.5
168	105th St. & 2nd Avenue	2 mo.	No. 35	2	1.5c.c.	213	98	115	52.7
43	129th St. & Amsterdam Avenue	2.5 mo.	No. 23	2	1.5c.c.	183	103	80	43.8
Man.									
43	135th St. & Brown Place	2.5 mo.	No. 35	2	1.5c.c.	397	257	140	35.2
Bx.									
186	145th St. & Broadway	2.0 mo.	No. 23	2	1.5c.c.	291	206	85	29.9
132	182nd St. & Wadsworth Avenue	2.0 mo.	No. 23	2	1.5c.c.	213	154	59	27.7
46	St. Nicholas Avenue & 157th Street	2.0 mo.	No. 35	2	1.5c.c.	302	226	76	25.1
157	127th Street and St. Nicholas Avenue	2.0 mo.	No. 35	2	1.5c.c.	253	199	54	21.3

from the results of our previous experience that most of these children will then given a negative reaction.

III. *Age incidence in Relation to Active Immunization Against Diphtheria.* The child population can be divided for purposes of active immunization into five distinct groups.

TABLE 2

Importance of Age in Active Immunization With Toxin-Antitoxin.

A. *Infants Under Six Months.*	
(a) Under three months	Children mostly immune (maternal). Do not develop an active immunity after toxin-antitoxin injections.
(b) Three to six months	Generally found to be immune (maternal). Toxin-antitoxin may be used, but it is not as effective as when used in the following age-group. A negative Schick test cannot be depended upon to indicate a permanent immunity.
B. *Pre-School Age.*	
(a) Six months to two years	Schick test can be omitted in this group, as the proportion of positive reactors is very high. A negative Schick reaction cannot be depended upon to indicate a permanent immunity. Strongly advisable to give toxin-antitoxin *to all these children.*
(b) Two to five years	Schick test may be used first. Positive reactions very high. Procedure can be greatly simplified by omitting Schick test and injecting *all these children with toxin-antitoxin.*
C. *Public School Age.*	
(a) Five to six years	Incoming classes (Kindergarten and 1A). Schick test may be used first. Positive reactions over 65 per cent. *Strongly advisable to inject all these children with toxin-antitoxin.*
(b) Six to fifteen years	Schick test and control test *should* be used *first.* Many negative pseudo-recations must be excluded. Toxin-antitoxin for Schick positive and positive combined reactors only.
D. *High School Age.*	
(a) Fifteen to nineteen years	Schick test and control test should be used *first.* Many negative pseudo-reactions must be excluded. Toxin-antitoxin for Schick positive and positive combined recators only.
E. *Adolescents and Adults.*	
(a) Nineteen years up	Schick test and control test should be used *first.* Many negative pseudo-reactions must be excluded. Toxin-antitoxin for Schick positive and positive combined reactors only.

Table 2 gives in a condensed form the indications for the Schick test and toxin-antitoxin immunization.

A. Infants under six months are protected temporarily in fully 85 to 90 per cent. by an inherited maternal antitoxic immunity against diphtheria. The inherited antitoxin not only protects these children, but it is also interferes with the development of an active immunity, when they are injected with toxin-antitoxin.[5] It is, therefore, important to wait until most of this inherited antitoxin has been eliminated before attempting to inject the children with the toxin-antitoxin.

B. Probably the most important period of life in which the toxin-antitoxin should be used is from *six months to two years.*[6] A negative Schick reaction in this group cannot be entirely depended upon, as the reaction later changes in many of these infants from negative to positive (loss of maternal immunity). It is therefore *strongly advisable* to omit the Schick test and inject *all children* in this group with the three doses of toxin-antitoxin.

To make certain that we reach all of the susceptible children, it is just as strongly advisable to inject also *all children from two to five years of age.* The negative Schick reaction in this age group is probably a permanent index of natural immunity. The proportion of susceptible children, however, is very high and the *omission* of the Schick test would simplify this important immunizing procedure for many physicians and thereby make it more certain that a greater number of them would recommend and use it. The simple subcutaneous injection of three doses of toxin-antitoxin would appeal to many physicians who are not thoroughly acquainted with the technique or the interpretation of the Schick test.

The high proportion of positive Schick reactions in children from six months to five years of age and the corresponding high morbidity and mortality from diphtheria in this group (80-85 per cent. of all diphtheria cases) are strong indications for the active immunization of *all children under five years of age.* The injections of toxin-antitoxin produce very little pain and discomfort in these young children because only very few are hyper-sensitive to the bacillus protein as shown by the small percentage of negative pseudo-reactions among them. The statement may even be definitely made that the younger the child the more tolerant it is to the injections of toxin-antitoxin.

At all ages it should be remembered that no individual can be pronounced immune to diphtheria until he has been proven to be so by the Schick test. Until this is done it is always a question in each case whether the particular child has responded to the toxin-antitoxin stimulation. For this reason the Schick test must never be omitted as a final part of the procedure of active immunization against diphtheria.

The facts indicate that the solution of the diphtheria problem depends upon just such a general active immunization of all children of pre-school age. Compulsory immunization would be a great forward step in this direction, if public opinion could be converted by educational propaganda to the advisability of such a step.

C. The children of school age can be divided into two groups: (a) the entering classes and (b) the higher grades.

(a) The entering classes comprise in the educational system of New York City the Kindergarten and 1-A Grades. This group is of special interest to the health official, as it is through these classes that he is enabled for the first time to exercise his influence more directly upon the young children of the community. For the reasons stated above and until we shall have established the principle of actively immunizing *all* children of pre-school age against diphtheria, it will be advisable to inject *all children of the incoming classes* in our public schools with toxin-antitoxin. The omission of the Schick test will simplify the work for the school physician or health officer, who will then be more apt to recommend and use the toxin-antitoxin. If this procedure is followed, there will be created within a few years a diphtheria immune school population. Until we can do this as a routine we shall have to take into account for the present at least the many susceptible children who are now in the higher grades.

(b) In the second group, Grade 1-B and upward, the Schick test and control test should be used before immunization for the following reasons: 1. In many schools the number of positive Schick reactions may not be more than 16 to 25 per cent. In such schools by a preliminary Schick test many children can be saved from getting unnecessary injections of toxin-antitoxin. 2. From 10 to 25 per cent. of children of school age give a negative pseudo-reaction. These children are not only immune to diphtheria, but they are the very ones who would have *severe local and constitutional reactions* after injections of toxin-antitoxin.

School children who give a positive or positive combined reaction should receive the three injections of toxin-antitoxin. Children who have a positive combined reaction will generally show moderate or fairly severe local and constitutional symptoms, but most of these symptoms subside after two or three days. Over 50,000 injections of toxin-antitoxin, which we have given during the past four months, we have not seen a single serious result or a single infection. This statement must be emphasized as the swelling of the arm at the site of injection leads some of the parents to think that the child has "blood poisoning."

D. and E. Children over fifteen years of age and adults should always have the Schick test and control test applied before immunization to determine their susceptibility to diphtheria. It is in these individuals that we find the more marked forms of negative pseudo-reaction which should be identified as such and not mistaken for positive or positive combined reactions. It may be left optional with the adult individual who has a positive combined reaction whether or not he should receive the toxin-antitoxin.

For bibliography see the original article.

THE SCHICK TEST PERFORMED ON MORE THAN 150,000 CHILDREN IN PUBLIC AND PAROCHIAL SCHOOLS IN NEW YORK (MANHATTAN AND THE BRONX).

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Abraham Zingher

Performance of the Schick test and active immunization with toxin-antitoxin were started on a large scale in the public schools of New York during the spring of 1921. Since then the work has been continued under the general direction of Dr. Park, and nearly all the children in public and parochial schools in Manhattan and the Bronx have been tested by myself and my group of assistants. In Brooklyn and Queens the work is being carried out by Dr. Schroder and her associates. The following report represents the tabulated summary of the Schick test on more than 150,000 schoolchildren in Manhattan and the Bronx.

A. The Schick Test and Control Test: Dilutions for the Test

The Schick test was made on the right forearm of each child and the control test with heated toxin on the left forearm. The reactions were read from the third to the seventh day after the test, the day of reading depending on how the work could be arranged in the schools.

We consider the proper dilution of the toxin one of the most important elements in the accuracy of the test. When large numbers of children are to be tested, the use of unheated bulk toxin for the Schick test and of heated bulk toxin for the control is recommended as the most accurate and economical method for carrying on the work. It is practically the only way to prepare a large amount of a *uniform* toxin dilution and to be certain of the accuracy of the results as regards one of the essential elements in the test. From an economical point of view this method is most important as we can apply the Schick test in this way to more than 3,000 children, and the expense for the material used will amount to less than one dollar. If proper precautions are taken in making these dilutions as recommended in a previous publication,[1] there will be no danger that dilutions that are too toxic will be prepared. Certainly, health officers, school physicians and general practitioners that have to test large numbers of children should be able to make the proper dilutions without the fear of any unnecessary risks.

B. Susceptibility to Diphtheria by Age Periods

In Table 1 and in the succeeding tables the percentages reported are significant in that they are based upon large numbers of tested individuals. Statistics regarding susceptibility and immunity to diphtheria and conclusions to which they lead should be founded on tests that are accurately carried out on many individuals. Conclusions based on a small number of tests are frequently liable to be misleading.

Table 1 gives a summary of the Schick tests carried out in the public and parochial schools in Manhattan and the Bronx. It shows the susceptibility by age periods of more than 150,000 children in a mixed population, as represented in the schools of New York. It is important to note that

TABLE 1.

THE SCHICK TEST IN THE PUBLIC AND PAROCHIAL SCHOOLS OF NEW YORK (MANHATTAN AND THE BRONX)

Age, Years	Total No. of Children Tested	Schick Positive Reactions	Schick Negative Reactions	Percentage of Schick Positive Reactions
5- 6	4,809	2,902	1,907	60.3
6- 7	13,754	6,944	6,810	50.4
7- 8	16,180	7,042	9,138	43.5
8- 9	17,126	6,278	10,848	36.6
9-10	18,066	5,826	12,240	32.2
10-11	18,057	5,308	12,749	29.3
11-12	17,994	5,075	12,919	28.2
12-13	16,253	4,327	11,931	26.6
13-14	14,138	3,271	10,867	23.1
14-15	9,650	1,906	7,744	19.7
15-16	4,861	869	3,992	17.8
16-17	369	68	301	18.4
Total	151,261	49,815	101,446	32.9

more than 60 per cent. of the children gave a positive Schick reaction upon entering school.

Table 2 shows the susceptibility to diphtheria of a large group of children between 6 and 12 months and between 1 and 14 years of age. In Group A are the children that were tested at the baby health stations. They represent a poorer class of the population. It is interesting to note that even in this group of the population there is a very high percentage of positive Schick reactions in children under 1 year, especially from 8 to 12 months of age. The susceptibility continues very high up to 3 years of age. It is probable that even in the poorer classes of the population the very young children are kept fairly well separated, and exposure to repeated infection is thus to a certain extent avoided. The percentage of positive reactions diminishes very rapidly after the age of 7 years.

TABLE 2.

GROUP A: THE SCHICK TEST IN BABY HEALTH STATIONS AND PLAY GROUNDS (MANHATTAN AND THE BRONX)

Age	No. of Children Total Tested	Schick Positive Reactions	Schick Negative Reactions	Percentage of Schick Positive Reactions
6- 7 months	53	30	23	56.6
7- 8 months	41	26	15	63.4
8- 9 months	62	52	10	83.8
9-10 months	58	54	4	93.1
10-11 months	61	53	8	87.0
11-12 months	34	31	3	91.1
1- 3 years	1,727	1,438	289	83.2
4- 6 years	1,328	779	549	58.6
7-10 years	903	330	573	36.5
11-14 years	235	53	182	22.5
Total	4,502	2,346	1,656	63.2

GROUP B: THE SCHICK TEST IN PUBLIC SCHOOL CHILDREN OF KINDERGARTEN AND IA CLASSES

5- 6 years	327	253	64	77.15
6- 7 years	933	632	301	67.73
Total	1,260	885	365	70.25

TABLE 3.

RESULTS OF SCHICK TEST AT PUBLIC SCHOOL 52 (MANHATTAN)

Age	Total No. of Children Tested	Schick Positive Reactions	Schick Negative Reactions	Percentage of Schick Positive Reactions
5- 6 years	19	16	3	84.21
6- 7 years	69	57	12	82.61
7- 8 years	114	83	31	72.81
8- 9 years	91	69	22	76.26
9-10 years	83	59	24	71.09
10-11 years	60	35	25	58.33
11-12 years	64	44	20	68.75
12-13 years	46	31	15	67.39
13-14 years	42	22	20	52.38
14-15 years	18	11	7	61.11
15-16 years	10	5	5	50.00
Total	616	432	184	70.13

In Group B the table gives by contrast the results in a series of twelve schools in which more than 77 per cent. of the children between 5 and 6 years of age had shown a positive reaction. These schools are located in the less congested sections of the city and attended by children of the more well-to-do. In this group are included a few schools in which from 85 to 90 per cent. of the children in the kindergarten and 1 A classes gave a positive Schick reaction. Table 3 gives the findings in one of these schools.

These figures emphasize the high proportion of susceptible children on admission to school and should be remembered in connection with the practical work of immunizing them against diphtheria. When too many difficulties are in the way of giving the preliminary Schick test to these young children the test could be omitted and the children simply injected with the three doses of toxin-antitoxin.

Table 4 gives the results of a more detailed study of the Schick tests on 60,100 children, which represent a part of the tests in Table 1. In this table the reactions are classified as positive and positive-combined among the non-immunes, and as negative and negative-pseudo among the immunes. The table brings out the following two interesting and important facts:

1. A considerable number of children of school age show negative-pseudo reactions. These reactions should be identified with accuracy by means of the control test. The mistake will thus be avoided of interpreting them as positive Schick reactions with the resulting error of giving the children unnecessarily the injections of toxin-antitoxin. It is in this very group that the toxin-antitoxin produces the most marked local and constitutional symptoms.

2. The negative-pseudo reactions were more than two and one-half times as frequent among the immunes as the positive-combined reactions among

the nonimmunes. This is additional evidence that exposure to infection with the diphtheria bacillus is an important factor in the development of the so-called "natural" antitoxic immunity. Such exposure produces not only an antitoxic immunity, but also seems to sensitize many individuals to the protein substance of the diphtheria bacillus. This sensitization appears in the form of negative-pseudo reactions in the Schick test and control test.

TABLE 4.

THE SCHICK TEST IN THE PUBLIC SCHOOLS OF NEW YORK (MANHATTAN AND THE BRONX): SUSCEPTIBILITY TO DIPHTHERIA BY AGES

Age Years	Total Tested	Nonimmune			Immune			Percentage of Nonimmune			Percentage of Immune		
								Of Total Tested	Percentage of Positive Combined Reactions		Of Total Tested	Percentage of Negative-Pseudo Reactions	
		Schick Positive Reactions	Positive-Combined Reactions	Total Schick Positive Reactions	Schick Negative Reactions	Negative-Pseudo Reactions	Total Schick Negative Reactions		Of Total Tested	Of Total Schick Positive		Of Total Tested	Of Total Schick Negative
4– 5	30	21	0	21	8	1	9	70.0	0.0	0.0	30.0	3.0	11.0
5– 6	1,575	975	21	996	508	71	579	63.2	1.3	2.0	36.7	4.0	12.0
6– 7	5,377	2,586	61	2,647	2,393	337	2,730	49.2	1.0	2.0	50.8	6.0	12.0
7– 8	6,130	2,525	112	2,637	3,010	483	3,494	43.0	2.0	4.0	57.0	8.0	14.0
8– 9	6,539	2,176	187	2,363	3,484	692	4,176	36.1	2.8	8.0	63.9	10.0	16.0
9–10	7,071	2,029	125	2,224	3,802	1,045	4,847	31.4	2.7	9.0	68.6	14.0	21.0
10–11	7,205	1,867	197	2,064	3,997	1,164	5,141	28.6	2.7	9.0	71.4	16.0	22.0
11–12	7,344	1,901	222	2,123	4,063	1,158	5,221	28.9	3.0	10.0	71.1	16.0	22.0
12–13	6,405	1,608	212	1,820	3,748	837	4,585	28.4	3.0	11.0	71.6	13.0	18.0
13–14	6,252	1,188	227	1,415	3,466	1,371	4,837	22.6	3.0	16.0	77.4	22.0	28.0
14–15	4,147	669	123	792	2,520	832	3,352	19.1	3.0	15.0	80.9	20.0	25.0
15–16	1,760	270	50	320	1,068	372	1,440	18.1	3.0	16.0	81.9	21.0	26.0
16–17	247	38	11	49	154	44	198	19.8	4.0	23.0	80.1	18.0	22.0
17–18	18	2	0	2	14	2	16	11.1	0.0	0.0	88.9	11.0	12.0
Total	60,100	17,855	1,618	19,473	32,235	8,409	40,624	32.6	2.7	8.3	67.4	14.0	20.7

The racial factor may also be noted in these tables.[1] The schools in which there is the highest percentage of positive Schick reactions are attended largely by native born children of American parents. The schools showing the lowest percentage of positive Schick reactions are those attended to a great extent by children of Italian parents. The other schools with percentages varying between these extremes are attended by children of more recently immigrated Jewish, Russian, Polish, Czechoslovacian, Irish parents, etc., one or two of these nationalities frequently predominating in some of the schools. A table giving the percentage of positive Schick reactions among children in parochial schools is also interesting in showing that overcrowding and repeated exposure to infection are really more important than race as factors in natural immunity to diphtheria. The three schools in the Bronx in which the children gave the highest percentage of positive Schick reactions are attended largely by children of Irish extraction. On the other hand, some of the schools in Manhattan which show a very low percentage of positive reactions, as for instance St. Bernard's, are also attended mostly by children of Irish parentage. It is significant to note that the school with the lowest percentage of positive Schick reactions, St. Patrick's, is located in the most congested section of the city, in which the population is nearly all Italian.

The results stated in these tables correspond closely to those that were reported in a previous communication in a series of forty-four schools in Manhattan and the Bronx.

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[1] See original paper.

Table 5 shows the very high percentage of positive Schick reactions in public schools located in small suburban towns, attended to a large extent by children of a more well-to-do class. Many of those that gave negative reactions were children of foreign parents recently settled in town.

TABLE 5

THE SCHICK TEST IN THE PUBLIC SCHOOLS OF METUCHEN, N. J.; CALDWELL, N. J.; VERONA, N. J., AND CEDAR GROVE, N. J.

Age, Years	Nonimmune			Immune			Total Tested	Percentage of Schick Positive Reactions
	Schick Positive Reactions	Positive-Combined Reactions	Total Schick Positive Reactions	Schick Negative Reactions	Negative Pseudo Reactions	Total Schick Negative Reactions		
5– 6	62	0	62	7	0	7	69	89.8
6– 7	73	0	73	11	1	12	85	85.8
7– 8	82	0	82	13	1	14	96	85.4
8– 9	93	0	93	22	2	24	117	79.5
9–10	75	0	75	24	2	26	101	74.2
10–11	61	0	61	21	9	30	91	67.0
11–12	74	1	75	19	4	23	98	76.5
12–13	62	2	64	20	4	24	88	72.7
13–14	64	2	66	17	7	24	90	73.3
14–15	22	1	23	11	2	13	36	63.9
15–16	41	2	43	10	4	14	57	75.4
16–17	39	1	40	11	5	16	56	71.4
Total	748	9	757	186	41	227	984	76.9

Table 6 gives the results of the Schick test in four public schools located in Union Hill, N. J. The school children can be divided fairly evenly into two groups, those of American born and those of foreign born parents. Among the latter we found a much higher proportion of negative Schick reactors.

D. SUSCEPTIBILITY TO DIPHTHERIA BY SEX

In our previous work with the Schick test, covering a period of more than nine years, we have generally found a larger number of positive reactions among females than among males.

TABLE 6.

THE SCHICK TEST IN THE PUBLIC SCHOOLS OF UNION HILL, N. J.

Age, Years	Schick Positive Reactions	Schick Positive Combined Reactions	Total Schick Positive Reactions	Schick Negative Reactions	Schick Negative Pseudo Reactions	Total Schick Negative Reactions	Total Tested	Percentage of Schick Positive Reactions
5– 6	46	0	46	22	1	23	69	66.66
6– 7	49	1	50	42	3	45	95	52.63
7– 8	59	4	63	60	9	69	132	47.72
8– 9	55	5	60	55	14	69	129	46.51
9–10	44	5	49	62	22	84	133	36.84
10–11	43	6	49	59	30	89	138	35.50
11–12	40	5	45	63	31	94	139	32.37
12–13	35	7	42	73	30	103	145	29.00
13–14	56	9	65	77	53	130	195	33.33
14–15	46	10	56	46	30	76	132	42.42
15–16	14	7	21	24	11	35	56	37.50
16–17	6	0	6	6	2	8	14	42.85
Total	493	59	552	589	236	825	1,377	40.00

Table 7 shows a definitely higher percentage of positive Schick reactions among girls than among boys. Among the girls there is also a higher proportion of negative-pseudo and positive-combined reactions.

TABLE 7.

COMPARISON OF SUSCEPTIBILITY TO DIPHTHERIA BETWEEN BOYS AND GIRLS IN NEW YORK PUBLIC SCHOOLS (PUBLIC SCHOOLS 188, 39, 83, 159, MANHATTAN)

Age, Years	Boys		Girls		Percentage of Schick Positive Reactions		Percentage of Negative-Pseudo Reactions of Immunes		Percentage of Positive-Combined Reactions of Nonimmunes	
	Total	Schick Positive Reactions	Total	Schick Positive Reactions	Boys	Girls	Boys	Girls	Boys	Girls
5– 6......	74	35	60	27	46.0	45.0	17.5	21.2	0	0
6– 7......	195	74	207	84	37.8	40.6	8.2	24.4	4.1	1.0
7– 8......	248	83	244	93	33.4	38.1	11.5	23.2	3.6	5.3
8– 9......	277	76	219	68	27.4	31.5	15.0	22.2	5.2	11.8
9–10......	393	86	281	82	21.9	39.2	21.5	30.6	7.0	15.8
10–11......	675	147	264	62	21.8	23.4	24.2	38.1	12.7	16.1
11–12......	782	153	299	72	19.5	24.1	26.8	31.7	18.3	15.3
12–13......	783	116	479	99	14.8	20.7	26.5	36.0	14.6	21.2
13–14......	740	91	497	100	12.3	20.1	26.6	35.7	19.7	23.0
14–15......	500	65	361	67	13.0	21.3	27.6	29.5	23.1	19.4
15–16......	224	17	155	21	7.5	13.4	18.3	36.5	0.0	23.3
16–17......	31	2	21	3	6.4	14.3	27.6	33.3	0.0	33.3
Total....	4,922	945	3,087	778	19.2	25.3	23.7	32.3	11.9	14.2

In Table 8 are given the results of the Schick test on more than 6,000 adult inmates of an insane asylum. The table brings out the fact that even among those who are far advanced in years there is definite though small percentage of positive Schick reactors. It shows that repeated exposure even

TABLE 8.

THE SCHICK REACTION IN ADVANCED AGE GROUPS (MANHATTAN STATE HOSPITAL)

Age, Years	Schick Positive Reactions	Schick Positive Combined Reactions	Total Schick Positive Reactions	Schick Negative Reactions	Schick Negative Pseudo Reactions	Total Schick Negative Reactions	Total Tested	Percentage of Schick Position Reactions
Males—								
10–20...........	12	1	13	57	18	75	88	14.7
20–30...........	47	8	55	551	99	650	705	7.8
30–40...........	45	11	56	580	110	690	746	7.5
40–50...........	28	2	30	418	69	487	517	5.8
50–60...........	21	1	22	292	20	312	334	6.5
60–70...........	5	0	5	142	4	146	151	3.3
Over 70.........	2	0	2	59	1	60	62	3.2
Total...........	160	23	183	2,099	321	2,420	2,603	7.0
Females								
10–20...........	9	2	11	60	9	69	80	13.7
20–30...........	76	15	91	376	81	457	548	16.6
30–40...........	89	13	102	514	126	640	742	13.7
40–50...........	58	12	70	546	87	633	703	9.9
50–60...........	34	3	37	466	83	549	586	6.3
60–70...........	30	1	31	446	34	480	511	6.6
Over 70.........	8	0	8	107	4	111	119	6.7
Total...........	304	46	350	2,515	424	2,939	3,289	10.0

over a long period of years does not produce an antitoxic immunity in certain individuals. These figures help to explain why it is difficult to develop an active immunity in a small percentage of individuals even with several series of toxin-antitoxin injections. There is a partial or nearly total inability of the tissues in some people to respond to the immunizing effects of certain antigens. This resistance is probably hereditary in character.

The table also shows a higher percentage of positive Schick reactions among adult females than among males.

E. "Natural" Immunity to Diphtheria

In the preceding tables we have seen some of the important factors active in the development of spontaneous immunity to an endemic infectious disease. These factors consist of crowding and repeated exposure to infections with the diphtheria bacillus. In cities this organism is constantly present. From 2 to 3 per cent. of the inhabitants are carriers of virulent diphtheria bacilli. The high percentage of positive Schick reactions among the children of the more well-to-do and the low percentage among those of the poorer classes show the importance of overcrowding in the development of immunity. In the smaller communities and especially in rural sections where the people live more or less segregated, the percentage of positive Schick reactions is very high. In rural schools we have seen from 85 to 90 per cent. of positive Schick reactions. This is true not only for children but also for adults. In various city hospitals we have tested groups of nurses, many of whom had only recently come to the city from smaller communities, and have found that among them from 50 to 75 per cent. gave a positive reaction to the Schick test. Similar observations were reported to me in a personal communication by Dr. Charles W. Kidder, District Health Officer of Woodstock, Vermont. He gave the Schick test to 2,030 children and found in rural towns, with a very high percentage of native born children, as high as 96 per cent. of positive reactors. In the larger villages on the railroad and in villages containing a large percentage of foreign population, he obtained correspondingly fewer positive Schick reactors, the lowest percentage being 60. Of eighty-seven teachers between the ages of 20 and 60 years, 82 per cent. gave a positive reaction.

Table 9 is an interesting study of the effect of close contact in an institution. It gives the results of the Schick test in an institution in which most of the children came from New York City. Under "Institution" children are included those that have been at the institution three months or longer; under "Preventorium" children those who have been there less than three months.

Among the "Preventorium" children, twenty-six of a total of seventy-eight, or 33.3 per cent., gave a positive Schick reaction. This is in striking contrast to the "Institution" children of whom only twenty-eight of a total of 520, or 5.4 per cent., gave positive reactions. Even among the twenty-eight positively reacting "Institution" children, fully twenty had been in the institution only from three months to two years. Frequent contact in the institution explains these results.

Dudley has recently reported the results of the Schick test in an institution in which diphtheria had been prevalent and had resulted in a large number of carriers. This study furnishes additional evidence that exposure to infection with the diphtheria bacillus is an important factor in the development of contact or so-called "natural" immunity.

TABLE 9.

THE SCHICK TEST AT ST. AGATHA'S HOME, NANUET, N. Y.

Age, Years	Institution Children						Preventorium Children (Less Than Three Months in the Institution)			
	Total Tested	Schick Negative Reactions	Negative-Pseudo Reactions	Total Immune	Schick Positive Reactions	Percentage of Schick Positive Reactions	Total Tested	Schick Negative Reactions	Schick Positive Reactions	Percentage of Schick Positive Reactions
2– 3..........	8	4	0	4	4	**50.0**	0	0	0	**0**
3– 4..........	13	8	0	8	5	**38.5**	0	0	0	**0**
4– 5..........	21	16	0	16	5	**23.8**	6	2	4	**66.6**
5– 6..........	22	15	1	16	6	**27.3**	6	4	2	**33.3**
6– 7..........	53	42	2	44	9	**17.0**	7	4	3	**43.0**
7– 8..........	36	25	6	31	5	**13.9**	16	9	7	**43.7**
8– 9..........	44	35	7	42	2	**4.6**	9	6	2	**33.3**
9–10..........	54	44	8	52	2	**3.8**	11	10	1	**9.0**
10–11..........	63	59	3	62	1	**1.6**	9	6	3	**33.3**
11–12..........	59	46	10	56	3	**5.0**	8	6	2	**25.0**
12–13..........	67	51	13	64	3	**4.5**	2	1	1	**50.0**
13–14..........	50	37	9	46	4	**8.0**	3	3	0	**0.0**
14–15..........	38	30	6	36	2	**5.3**	1	1	0	**0.0**
15–16..........	15	11	4	15	0	**0.0**	0	0	0	**0.0**
Total........	543	423	69	492	51	**9.4**	78	52	26	**33.3**
Total*.......	520	...	...	492	28†	**5.4**	...	...	...	**33.3**

* Excluding twenty-three children giving a positive reaction who had been less than three months in the institution.

† Of the twenty-eight children giving a positive reaction, twenty had been in the institution between three months and two years.

Without going too far astray one could probably generalize from "natural" immunity against diphtheria to "natural" immunity against some of the other infectious diseases. Such immunity is seen in a large group of city born and city bred people who have never had these infections. Russell has drawn our attention to the fact that, during the recent war, epidemics of infectious diseases swept, as a rule, through camps in which the soldiers were recruited from the smaller towns and villages and from rural sections of the states. Many city children are often found to be relatively immune to some of these infectious diseases although they give no history of having had the infections. Herrman's observations on immunity to measles also bear on this point. It has, of course, been often assumed that repeated exposure to infectious diseases, or to the carriers of their viruses or bacteria, is the chief factor in "natural" immunity; but so far it is only in diphtheria that we have been able to demonstrate this with any degree of scientific accuracy.

Summary and Conclusions

1. More than 150,000 children have been given the Schick test during the last two years in the public and parochial schools of the boroughs of Manhattan and the Bronx.

2. A high percentage of positive Schick reactions is found among the children in the entering classes in the kindergarten and 1 A grades. This varies in different schools from 60 to 85 per cent. The toxin-antitoxin injections could, therefore, be given to these young children without the preliminary Schick test.

3. The schools located in the less congested sections of the city and attended by children of the more well-to-do show, as a rule, from 100 to 200

per cent. more Schick positive reactors than those located in the crowded parts and attended by children of the poorer classes.

4. Repeated exposure to infection with the diphtheria bacillus is probably the most important factor in the development of "natural" immunity to this disease. Racial and hereditary family factors, although less important, also play a rôle.

5. Girls show a somewhat larger percentage of positive Schick reactions than boys. They also show more negative-pseudo and positive-combined reactions. This holds true also for adults.

6. The high percentage of positive Schick reactors among young children of preschool age may, under certain conditions, render it advisable to simplify the procedure of immunization by omitting the preliminary Schick test. The children are simply injected with three doses of toxin-antitoxin. Under these circumstances, however, the children should not be pronounced immune to diphtheria until a supplementary Schick test, performed six months later, proves negative.

7. Diphtheria is the foremost fatal disease in children from 3 to 5 years of age, and ranks third among the fatal diseases of children from 1 to 3 years of age. Only diarrhea and pneumonia precede diphtheria in the mortality tables. The dangerous laryngeal form occurs in the young children. It is for these reasons that physicians should urge parents to take advantage of these newer methods to protect their young children against diphtheria. In fact, active immunization against diphtheria should become as much of a routine for all children between 6 months and 6 years as vaccination is against smallpox today.

For bibliography see the original article.

COMPARATIVE MERITS OF NEW AND OLD PREPARATIONS OF DIPHTHERIA TOXIN-ANTITOXIN

Results of Preliminary Tests with Toxoid

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When the New York City Health Department laboratory workers first undertook to estimate the value of toxin-antitoxin in the practical immunization of children against diphtheria in 1914, it was essential to utilize an effective preparation. The ingredients of importance in such a preparation other than the antitoxin are the specific toxin that causes the production of antitoxin and the nonspecific products of bacterial growth and dissolution occurring in the cultures in which the toxin is produced. It is these nonspecific products that cause the local disturbance in sensitive persons. The antitoxin should be added in an amount just sufficient to lessen the excessive avidity of the toxin for the tissues and so prevent painful reaction, but the amount of antitoxin should not be enough to rob the toxin of its immunizing effect.

For a number of years previously, numerous animal tests had been carried out by Park with a mixture containing three L plus doses of toxin to each cubic centimeter, and sufficient antitoxin to reduce the toxicity so that an injection of 1 c.c. caused paralysis in the guinea-pig in about twenty-five days.

As we had studied this preparation and knew it to be efficient and safe, we determined to use it for the immunizing injections. The results were in every way satisfactory except for the rather excessive local reaction. This mixture became our standard preparation. However, with the increasing utilization of toxin-antitoxin not only in New York City but also throughout the country, we felt constrained to endeavor to remove as much as possible of the irritating substances in the mixture.

Dr. Banzhaf of our laboratory attacked this problem, but neither he nor other chemists have been able to separate the useless and annoying bacterial substances in the culture fluids from the valuable specific toxin. Despairing of success in eliminating the extraneous proteins from the specific toxin, we attempted to achieve our object by lessening the amount of both toxin and antitoxin in the mixture and so also the amount of the proteins. This was suggested to us by the reports of the Schick retests by Zingher on a number of children in the borough of Manhattan, and by Schroder on children in the borough of Brooklyn. A toxin of double the standard strength had been used on these children. The immunizing results, instead of exceeding those obtained with the standard preparation, were merely similar. A doubling the amount of toxin in the dose did not improve the effectiveness of the mixture, it seemed reasonable to try the effect of reducing the amount of toxin. We therefore tried mixtures in which the amount of toxin was one third, one sixth, one thirtieth and one sixtieth of the standard dose. The preparation (0.1 L + toxin) containing one thirtieth of the original toxin was finally selected as probably the best. A preliminary report of the observations on 490 children on whom this mixture was employed by Schroder was given at the meeting of the American Public Health Association[1] in October, 1922. The results are shown in Tables 1 and 2.

Because of these favorable results, it was decided to make the one-thirtieth (one-tenth L plus toxin) preparation the standard, instead of the three L plus mixture. Since then no other mixture has been used in our work in immunization except when we wished to make comparisons. The results of a comparative test in adults are given in Table 3.

TABLE 1.

THE ANTITOXIN DEVELOPMENT PRODUCED BY THREE INJECTIONS OF MIXTURES* HAVING DIFFERENT AMOUNTS OF TOXIN PARTIALLY NEUTRALIZED BY ANTITOXIN

Amount of original toxin in 1 cc. of Mixture	Number of children receiving 3 injections	Per-Cent of nonimmunes shown to be immune on Schick retest four months later
0.1 L+† (4 lethal doses)......................	490	90
0.5 L+ (20 lethal doses)......................	304	95
3.0 L+ (120 lethal doses)....................	318	92
5.0 L+ (200 lethal doses)....................	487	85

*All mixtures caused severe paralysis in guinea-pigs followed usually by death in eighteen days among those animals that received 1 c.c.; with 5 c.c. doses, death followed in ten days.

†The mixture is made by adding three-fourths unit of antitoxin to one L plus dose of toxin. The toxin and antitoxin should be diluted in cold water and the two solutions mixed immediately. If the toxin is diluted in water at room temperature, it deteriorates rapidly.

TABLE 2.

COMPARISON AS TO THE AMOUNT OF LOCAL AND CONSTITUTIONAL REACTION CAUSED BY THE NEW AND OLD PREPARATIONS IN CHILDREN

	New Preparation 0.1 L+* %	Old Preparation 3 to 5 L+ %
No local reaction..................................	25	0
Slight local reaction..............................	64	41
Moderate local reaction..........................	11	37
Marked local reaction............................	0	22
Among those showing marked reactions there was a rise of temperature of from 1 to 3 degrees F., and other constitutional symptoms in............	0	6

*If the 0.1 L plus preparation is underneutralized more than the amount advised, there will be a local reaction from the excess of toxin.

The immunizing results in adults with the one-tenth L plus and the three L plus preparations were similar to those previously obtained in children (Table 1). The local reactions to both preparations were somewhat more severe than with similar preparations in the children (Table 2), but the amount of local reaction following injections of the one-tenth L plus mixture was strikingly less than that following the injections of the three L plus mixture. The slightly lesser immunity response of the one-tenth mixture is attributed to the fact that the toxin was slightly less neutralized. One group of adults received three doses of only 0.5 c.c. each instead of 1 c.c. of the mixture. Those individuals who received the smaller injections had nearly as much local reaction without as great an immunity response.

Local and Immunity Reactions Produced by Toxin and by Toxoid

In the first experiments on antitoxin production in animals by von Behring and his associates, the diphtheria toxin was modified by exposure to iodin so as to reduce its toxicity. The action of the iodin or of other agents that cause a lessening of the toxicity and reduce the toxin to a form called toxoid produced an effect on the toxin somewhat similar to the action of slight amounts of antitoxin. We thought it would be of great interest to compare the results of such injections with those obtained by the injection of the toxin-antitoxin. It happened that at this time we had a large number of adults to immunize. This enabled us to use the different preparations under comparable conditions. We utilized a toxin that had been stored after being diluted with salt solution and had thus been reduced to toxoid. This was carefully tested in guinea-pigs. We arranged also to give a small group three injections of standard toxin at the same time. The amount of toxin chosen was that used in the Schick test. Through a misunderstanding, the first dose given was four times this amount. The results of these various preparations are shown in Table 3.

The local reaction of an appreciable dose of standard toxin alone was too severe to make it an acceptable preparation for general use. On the other hand, as shown in Table 3, the deteriorated toxin (toxoid) gave a fairly satisfactory immunizing response with remarkably little local reaction. We hope in the near future to carry on further tests with the diphtheria toxoid preparations. It is evident that fresh toxin must either be modified by antitoxin or partially broken down by some chemical or physical agency, so as to lessen the avidity with which it attacks the tissues; otherwise, the local reaction of the tissues is so severe that the amount of toxin injected has to be reduced to a dose so small that the immunity response in those who have no antitoxin will be very much less than when the usual dose of toxin-antitoxin or toxoid is used.

TABLE 3.

LOCAL REACTION ON SECOND DAY AND IMMUNITY RESPONSE AT THE END OF THREE AND ONE-HALF MONTHS FROM TOXIN-ANTITOXIN, UNMODIFIED TOXIN AND TOXOID*

Preparation Used	Three doses of, cc.	Adults tested	Severity of Local Reaction Very marked, %	 Marked, %	 Moderate, %	 Slight, %	 None, %	Immunity response after 3½ months Schick negative %
Toxin-antitoxin:†								
3.0 L+ in 1 c.c.	1	261	13	36	22	12	17	87
0.1 L+ in 1 c.c.	1	117	..	10	20	44	26	83
0.1 L+ in 1 c.c.	0.5	86	..	8	23	45	24	73
Freshly diluted standard toxin without antitoxin.								
0.15 M. L. D. in 1 c.c. 1st dose	1	29‡	3.5‡	24‡	45‡	24	3.5	41
0.025 M. L. D. in 1 c.c. (2d and 3d doses)	1	29	..	4	8	25	62	
Toxoid § (old and much deteriorated toxin)	1	211	..	3	10	37	50	70

* This table was given in the preliminary report read at the meeting of the Association of Pathologists and Bacteriologists in March, 1023.

† Average toxicity when injected in guinea-pigs weighing 250 gm.: 3 L plus in 1 c.c. = death in 23 days; 0.1 L plus in 1 c.c. = death in 30 days.

‡ The local reaction continued to increase after the second day instead of decreasing as it does with properly standardized toxin-antitoxin or toxoid.

§ One cubic centimeter caused late paralysis in guinea-pigs.

IMPORTANCE OF A SUITABLE DEGREE OF NEUTRALIZATION OF TOXIN IN TOXIN-ANTITOXIN MIXTURE

There has been considerable difference of opinion among investigators on this subject. Park and Banzhaf will soon make a final report on a large number of observations obtained from guinea-pig experiments. We report here the result of the retests on more than 2,000 children, on some of whom preparations of standard toxicity were used and on others preparations of somewhat insufficient toxicity (Table 4).

TABLE 4.

IMMUNITY RESULTS IN CHILDREN AFTER THREE INJECTIONS OF TOXIN-ANTITOXIN MIXTURES OF DIFFERENT DEGREES OF NEUTRALIZATION

Preparation No.	Toxicity in guinea-pigs: Dose	Toxicity in guinea-pigs: Result	Number of children injected	Immunity response after 4 to 6 months Schick negative, per cent.
1	1 c.c.	Death in about 17 days	131	92
	5 c.c.	Death in 4 to 5 days		
2	1 c.c.	Death in about 17 days	269	92
	5 c.c.	Death in 5 to 8 days		
3	1 c.c.	Death in about 21 days	796	91
	5 c.c.	Death in 5 to 6 days		
4	1 c.c.	Marked paralysis of animal followed by recovery	608	87
	5 c.c.	Death in about 17 days		
5	2 c.c.	Marked paralysis of animal followed by recovery	137	85
	5 c.c.	Death in about 22 days		
6	2 c.c.	Marked paralysis of animal followed by recovery	763	76
	5 c.c.	Death in about 25 days		
			2,704	

TABLE 5.

COMPARISON OF SCHICK RETEST RESULTS AT DIFFERENT INTERVALS AFTER IMMUNIZATION

Interval	Number of retests	Per cent. immune
3 to 3½ months	579	82.7
4 months	1,427	84
5 months	1,302	86.7
6 months	1,059	88
	4,367	

It is noticeable that, while the ideal preparations gave the best results, those preparations approaching considerably nearer to complete neutralization still yielded fair results. Therefore, although it is important to have preparations of a proper degree of toxicity, it is valuable to know that less toxic preparations will give in the majority of children sufficient antitoxic immunity response.

TABLE 6.

COMPARATIVE INTENSITY OF LOCAL REACTION TWENTY-FOUR AND FORTY-EIGHT HOURS AFTER TOXIN-ANTITOXIN INJECTIONS

		Number having greatest intensity of reaction at		
Greatest intensity of reaction	Total number of persons observed	24 hours	48 hours	Same at 24 and 48 hours
Very marked	23	..	4	19
Marked	31	6	9	16
Moderate	15	4	6	5
Slight	21	..	20	1
Totals	90	10	39	41

TIME INTERVAL BETWEEN INJECTION OF TOXIN-ANTITOXIN AND DEVELOPMENT OF ANTITOXIN IN SUFFICIENT AMOUNT TO INSURE IMMUNITY

It is necessary to make a retest for the Schick reaction if we desire to know positively whether the immunizing injections have given complete protection. Although it is desirable to make these retests as soon as possible, we defeat our purpose if they are made before the majority of the children have had time to react. The results obtained in about 4,000 retested children are given in Table 5.

These results indicate that although longer periods give somewhat more favorable results, retests may be made satisfactorily at any time after three months. The children tested at these intervals had received identical toxin-antitoxin preparations.

While the observations reported in this paper were being made, certain facts were noted that are of considerable clinical interest. These are the interval of time after the injection at which the intensity of the local toxin-antitoxin reaction is at its greatest, and the duration of the reaction. These observations are given in Tables 6 and 7.

TABLE 7

COMPARATIVE INTENSITY OF THE LOCAL REACTION AT END OF TWO, FIVE AND SIXTEEN DAYS, IN THREE HUNDRED CASES ASSEMBLED EQUALLY FROM PERSONS SHOWING MARKED, MODERATE AND SLIGHT REACTIONS

	Severity of reaction			
Time of inspection	Marked	Moderate	Slight	None
End of 2 days	100	..	..	..
End of 5 days	4	31	19	46
End of 16 days	..	..	7	93
End of 2 days	..	100	..	..
End of 5 days	..	11	26	63
End of 16 days	..	..	7	93
End of 2 days	..	..	100	..
End of 5 days	..	..	12	88
End of 16 days	..	..	..	100

As shown in the table, the local reaction subsides gradually after reaching its height on the first or second days.

CONCLUSIONS

The new preparation of toxin-antitoxin, with its smaller amount of toxin, possesses a distinct advantage over the earlier preparation recommended by us, in that the local inflammatory reaction sometimes developing after an injection is less severe and less frequent. Indeed, it is almost negligible in your children and infants. The two preparations have equal immunizing value.

The toxin-antitoxin mixture should not be too toxic; that is, too much underneutralized; if it is, the local reaction is disturbing. It should not be too nearly neutralized by antitoxin, for then the immunizing results are less. Safe limits as to toxicity are that 1 c.c. should not produce death in the guinea-pig in less than fifteen days on the average, and 2 c.c. should not fail to produce marked paralysis.

Antitoxin is added to diphtheria toxin solely for the purpose of robbing it of its irritant action on the local tissues and thus allowing a larger dose of the modified toxin to be injected. The same result may be obtained by partially breaking down the toxin to toxoid.

Unmodified standard toxin, such as is used in the Schick test, is so irritating that only minute doses can be used. Even these minute amounts have some immunizing value. Up to the present time, the addition of antitoxin to toxin gives us the best immunizing preparation.

The new preparation of toxin-antitoxin is somewhat less stable than the original preparation, but it is sufficiently stable to be used for six months after it has been properly standardized, if it is kept cool.

The use of the Schick test to determine the immunizing results of the toxin-antitoxin injections should not be approved of before the end of the third month, and preferably not before the end of the fifth month. This is because the immunizing response is a gradual one and requires for its completion a different period of time in each individual.

The local reaction which sometimes follows the toxin-antitoxin injections reaches its height on the first or second day, and then rapidly declines.

For bibliography see the original article.

IMMUNITY RESULTS OBTAINED WITH DIPHTHERIA TOXOID (MODIFIED TOXIN) AND ONE-TENTH L + MIXTURES OF TOXIN-ANTITOXIN IN THE PUBLIC SCHOOLS OF NEW YORK CITY (MANHATTAN AND THE BRONX)

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INTRODUCTION

The large and increasing use of subcutaneous injections of some form of modified toxin for purposes of active immunization, makes it important to utilize this toxin in the form in which it will produce the greatest amount of immunity, with minimum amount of deleterious after effect. It is important to grasp that in any preparation it is the diphtheria toxin or derivatives of it, and nothing else, that immunizes. Ehrlich, in 1895, discovered that when fresh diphtheria toxin deteriorates in strength a peculiar change takes place. The toxicity for the guinea-pig drops faster than its power to neutralize antitoxin. At a certain stage in deterioration the toxin in even large doses no longer causes death in animals, but it still neutralizes a considerable percentage of its original amount of antitoxin. Thus, if the preparation drops in toxicity from 100 minimum lethal dosage to 5 minimum lethal dosage per cubic centimeter, the original power to neutralize 1.0 unit of antitoxin still holds to the extent of neutralizing 0.1 of a unit; that is, while the toxicity has diminished to one-half of 1 per cent., the combining power has diminished only to 10 per cent.

Before Ehrlich had finished his researches, von Behring had already used this deteriorated toxin in his first experiments on the production of antitoxin. He utilized the addition of a small percentage of iodin tetrachlorid (1:500) to change the toxin so that he could immunize the animals safely. A little later it was discovered that by adding an amount of antitoxin to the toxin which would neutralize, or nearly neutralize, its toxicity in animals, its immunizing power still remained. The first modification of the toxin is called toxoid, the second toxin-antitoxin. While both of these modifications of toxin continued to be used in the immunization of animals, attention was concentrated more on the toxin-antitoxin, and it thus was the first to be used in the immunization of man. Thus it was that von Behring used it in his injections on human subjects.

When we began the work in New York, we also utilized a preparation of toxin-antitoxin because we were familiar with it. At first it was our view that a large amount of toxin almost completely neutralized would be more effective than a smaller amount less nearly neutralized. We therefore utilized a toxin-antitoxin containing in each dose 3 L + (120 minimum lethal dosage) of toxin and 3½ units of antitoxin. The immunizing results were satisfactory, but the local inflammatory reaction was occasionally severe in those sensitive to the nonspecific substances. We tried a preparation having nearly double the amount of toxin. This, to our surprise, gave no better results.

Comparison Between Old and New Type Mixtures of Toxin-Antitoxin

Table 1 shows that mixtures with the larger number of L + doses were not more efficient than those with the smaller number of L + doses, so long as the mixtures were equally toxic (under-neutralized). In fact, we noted that mixtures with the smaller number of L + doses per cubic centimeter, but showing greater toxicity for the guinea-pig, gave better immunity results than those with the larger number, which were more neutralized. This led one of us (P.) to make use of preparations containing less toxin and finally one containing but one-thirtieth of the amount of toxin, or 0. L + and 0.75 unit of antitoxin was adopted. The results were as satisfactory as with the other preparation, and the local reaction was much less. This has become the standard preparation in this country.

TABLE 1.

COMPARISON BETWEEN DIFFERENT MIXTURES OF TOXIN-ANTITOXIN VARYING IN TOXICITY AND NUMBER OF L + DOSES PER CUBIC CENTIMETER (ZINGHER)

		Action on Guinea-Pig (Tests by Banzhaf)		Results in Children				
Mixture	Composition of Toxin Antitoxin Mixture	Amount injected, C.c.	Result	Number doses	Amount of each dose, cc.	Total number Schick retested	Schick negative	Per cent. negative
41	6 L doses of toxin and 7 units of antitoxin	1.0	Partial paralysis in 10 days; recovery	3	1.0	985	570	57.8
		5.0	Paralysis; death in 15 days					
42	5 L doses of toxin and 5.8 units of antitoxin	0.5	Paralysis; death in 17 to 20 days	3	1.0	3,846	3,356	87.3
		1.0	Paralysis; death in 17 days					
		5.0	Death in 3 days					
43	3 L doses of toxin and 5.8 units of antitoxin	1.0	Paralysis in 16 days; recovery	3	1.0	142	137	95.9
		3.0	Paralysis; death in 17 days					
		5.0	Death in 3 days					

Table 2 shows the antitoxin development after three injections of mixtures having different amounts of toxin and all partially, but equally, neutralized by antitoxin.

TABLE 2.

THE ANTITOXIN DEVELOPMENT PRODUCED BY THREE INJECTIONS OF MIXTURES* HAVING DFFERENT AMOUNTS OF TOXIN PARTIALLY NEUTRALIZED BY ANTITOXIN (SCHROEDER)

Amount of original toxin in 1 cc. of mixture	Number of children receiving three injections	Percent of nonimmunes shown to be immune on Schick retest four months later
0.1 L+ (4 lethal doses)	490	90
0.5 L+ (20 lethal doses)	304	95
3.0 L+ (120 lethal doses)	318	92
5.0 L+ (200 lethal doses)	487	85

* All mixtures caused severe paralysis in guinea-pigs, followed usually by death in eighteen days among those animals that received 1 c.c.; with 5 c.c. doses, death followed in ten days.

Table 3 shows the mixtures used, the effect of varying doses and of mixtures of varying toxicity on guinea-pigs, and the immunity results in children. It is interesting to note the high percentage of children who became immune with mixtures 69 and 65. The number of retested children is sufficiently large to be representative, and thus avoid the error so frequently associated with small numbers in diphtheria immunity work.

TABLE 3.

COMPARISON OF RESULTS WITH DIFFERENT MIXTURES OF TOXIN-ANTITOXIN CONTAINING FROM ONE-TENTH TO ONE-TWENTIETH L + PER CUBIC CENTIMETER RELATION BETWEEN ACTION ON GUINEA-PIG AND IMMUNIZING EFFICIENCY IN CHILDREN (ZINGHER)

			Action on Guinea-Pig (Tests by Banzhaf)			Results in Children			
Mixture	Number L+ in each cc.	Number M.L.D. in each cc.	Amount Injected, cc.	Result	Number doses	Amount, cc.	Total number Schick retested	Schick negative	Per cent. negative
69	1/10	4	1.0	Partial paralysis; recovery	3	1.0	221	213	96.4
			3.0	Paralysis; death in 25 days					
			5.0	Death in 4 days					
65	1/10	4	1.0	Death in 8 days	3	1.0	900	814	94.0
			5.0	Death in 6 days					
67	1/10	4	1.0	Paralysis; death in 22 days	3	1.0	61	53	86.9
			3.0	Death in 9 days					
			5.0	Death in 3 days					
52	1/10	4	2.0	Paralysis in 21 days; recovery	3	1.0	384	342	89.0
			3.0	Paralysis in 17 days; recovery					
			5.0	Paralysis in 17 days; recovery					
70	1/10	4	1.0	Partial paralysis; recovery	3	1.0	55	48	87.4
			3.0	Paralysis; recovery					
			5.0	Paralysis; death in 21 days					
53	1/10	4	1.0	Paralysis; death in 21 days	3	0.5	28	24	85.7
			3.0	Death in 3-5 days	3	0.25	31	24	77.4
			5.0	Death in 3-5 days					
56	1/20	2	1.0	No local effect; no paralysis; recovery	3	1.0	165	97	59.4
			3.0	Paralysis in 21 days; death in 35 days					

LOCAL AND CONSTITUTIONAL REACTIONS PRODUCED BY NEW AND OLD TYPE MIXTURES OF TOXIN-ANTITOXIN

The local and constitutional reactions obtained with mixtures of toxin-antitoxin are due to two different substances in the mixtures.

(*a*) In the old type mixtures, containing from 3 to 6 L + doses of toxin per cubic centimeter, there was a considerable amount of autolyzed bacillary substance and other kinds of portein derived from the broth culture itself, which gave rise in sensitive persons to the most marked forms of local and constitutional reactions. These reactions were of an anaphylactic type and generally occurred in hypersensitive persons, who had given a negative pseudo reaction or positive combined reaction. Both the local and constitutional reactions reached their height within twenty-four hours, gradually disappeared in from two or three days, although they persisted at times for from four to six days. The local reaction varied from an area of redness and induration the size of a fifty cent piece to an extensive infiltration involving one-half, or more, of the arm. There was pain, tenderness and slight glandular enlargement in the axilla. Fever was present, the temperature ranging from 100 to 104 F. A few of the persons showing simple positive Schick reactions also had slight local and constitutional disturbances, but the more marked reactions to the toxin-antitoxin were only found in those who had shown themselves to be hypersensitive to the proteins of the mixture by their negative pseudo reactions or positive combined reactions.

(*b*) With the new type of mixture (one-tenth L +) the local and constitutional effects due to the reacting protein were to large extent eliminated. but the local effect of the larger amount of free toxin present in some of these preparations became more evident. We noticed, following the subcutaneous injections, especially with mixtures of which 1 c.c. caused death of the guinea-pig in from six to eight days and 3 c.c. in from three to five days, local reactions that were slow in appearing, but much more lasting than the protein reactions. These reactions were characterized by local induration and redness, which gradually faded into a brownish-red area of discoloration. The area had a dry, wrinkled, desquamating appearance and resembled on inspection a large positive Schick reaction. With one of these mixtures we even noticed in a few children a superficial necrosis of the skin. This local reaction was due to a large extent to the free toxic fraction of the mixture.

The majority of the mixtures of toxin-antitoxin used in the schools were less toxic, and corresponded more to the degree of underneutralization given for a standard preparation. With such mixtures the local reactions were only slight and often absent. It was possible, in fact, to inject several hundred school children with these new type mixtures during a day's work without hearing subsequent complaints about sore arms, or the necessity of the children remaining away from school for a day or two. Constitutional symptoms, such as fever, were practically absent. This was noticeable even after injecting the more toxic preparations, and was in striking contrast to the disagreeable effects seen with the 3 to 6 L + mixtures in from 5 to 20 per cent. of school children.

Equally suggestive of the value of toxoid as a good immunizing agent were the results obtained by Zingher with the old type mixtures (from 3 to 5 L + doses per cubic centimeter) of toxin-antitoxin. Table 4 indicates that these mixtures did not have their immunizing value impaired to any extent

TABLE 4.

RESULTS OBTAINED WITH FRESH MIXTURES AS COMPARED WITH OLDER MIXTURES OF TOXIN-ANTITOXIN CONTAINING FROM THREE TO FIVE L + TOXIN PER CUBIC CENTIMETER (ZINGHER)

			Action on Guinea-Pig (Tested by Banzhaf)		Results in Children			
Mixture	Number L + in each cc.	Number M.L.D. in each cc.	Amount injected cc.	Result	Number doses toxin-antitoxin	Total number Schick retested	Schick negative	Per cent. negative
44	..	...	1.0	Death in 6 days	3	1,080	1,047	97.0
Fresh	3	120	5.0	Death in 3 days				
44*	3	120	1.0	Slight local effect; death from pneumonia	3	142	137	96.4
Old	..	...	3.0	Moderate local effect; death in 12 days				
			5.0	Moderate local effect; death in 9 days				
42	5	200	0.5	Paralysis; death in 17-20 days	3	3,846	3,356	87.2
			1.0	Paralysis; death in 17 days				
Fresh	..	...	5.0	Death in 3 days				
42*	5	200	1.0	No local effect; no paralysis, recovery	3	364	361	99.1

* These mixtures were kept at icebox temperature for seven months.

after standing for several months, and losing nearly all their toxicity for the guinea-pig. The slight excess of free toxin being changed into toxoid, these favorable results indicated that toxoid could give rise to antitoxic antibodies and that it was probably an important factor in active immunization. Dissociation of the neutral mixture of toxin-antitoxin after injection need not be considered of any importance, in view of the poor immunizing value of freshly neutralized or overneutralized mixture.

Immunity Results Obtained with Toxoid

The above results suggested that there were possibilities in the toxoid preparation that made it well worth while to submit it to further experimentation. For the time being our attention was mainly centered on the new one-tenth L + preparation of toxin-antitoxin. The article of Glenny and Hopkins, on using a toxoid with antitoxin instead of the toxin-antitoxin mixture regularly used, then appeared. A little later O'Brien, of the Burroughs, Wellcome & Company laboratories, in writing to one of us said that they were getting excellent immunity results in animals with toxoid made by the addition of 0.1 per cent. formaldehyd to toxin. He sent some of this preparation to us and asked if we would try it on human beings. Dr. Banzhaf immediately injected it into guinea-pigs and Dr. Sawhill injected a few nurses at Bellevue Hospital. The guinea-pigs, when retested three and four weeks later showed immunizing results as good, but not better, than with the toxin-antitoxin preparation.

Glenny, Pope and Hopkins, in an as yet unpublished communication entitled "Further Notes on Modification of Diphtheria Toxin," state that the amount of formaldehyde necessary to produce a given effect on a batch of toxin depends greatly on the aminonitrogen content. To a toxin of which the van Slyke nitrogen was 2.8 mg. per 10 c.c., 0.1 per cent. of formaldehyd was added; after keeping for twenty-four hours at a temperature of 37 C., an intradermic reaction was no longer obtained if the toxin was diluted more than 250 times. Another toxin, with a van Slyke figure of 19.6 mg., after similar treatment, gave a reaction when diluted 10,000 times; this toxin needed at least 0.3 per cent. formaldehyde to reduce the toxicity to the same level as that reached by adding 0.1 per cent. to the other toxin. In a given toxin the amount of modification produced by a given concentration of formaldehyd depends on the temperature and time of exposure.

After modification of toxin many of the tests for combining power can no longer be made. If sufficiently modified, the toxin no longer kills the guinea-pig and so the L + dose cannot be determined. Even the reacting dose on the guinea-pig's skin can no longer be estimated if the toxin is completely changed into toxoid. To obtain an indication of the combining power of such a modified toxin that is no longer capable of producing symptoms in a guinea-pig, Glenny, Hopkins and Pope have added an excess of antitoxin, and after standing the mixture for a number of hours, determined by titration with toxin the amount of free antitoxin left in the mixture. By making a similar experiment previously with the same toxin, which had not been modified, they were able to draw some interesting and important conclusions, indicated in Table 5.

In Table 5 the number of units combined represents the difference between the number of units of free antitoxin detected in the mixture and the number of units originally added. This number of units they call the total combining power. They found that the total combining power had not been markedly reduced by changing the toxin into toxoid, but that the affinity of the modified toxin for antitoxin had been greatly reduced. This is indicated in parts 2 and 3 of Table 5. Leaving the toxoid in contact with the antitoxin for only four hours, as was done in the case of the unmodified toxin, the total combining power was found to be much less; it required fully forty-eight hours for the complete saturation of the toxoid by antitoxin.

TABLE 5.

COMBINING PROPERTIES OF TOXIN AND OF MODIFIED TOXIN FOR DIPHTHERIA ANTITOXIN (GLENNY, HOPKINS AND POPE)*

	Combining time with antitoxin: Four hours at room temperature				
1. Toxin Not Modified:					
Number of units of antitoxin added to 1 cc. of toxin..	15	20	30	40	50
Number of units detected after four hours............	5	8	18	28	38
Number of units combined	10	12	12	12	12
Total combining power, 12 units per cc.					
	Four hours at room temperature				
2. Toxin Modified:					
Number of units of antitoxin added to 1 cc. of toxin..	15	20	30	40	50
Number of units detected after four hours............	10	14	22	31	42
Number of units combined	5	6	8	9	8
	Forty-eight hours at room temperature				
3. Toxin Modified:					
Number of units of antitoxin added to 1 cc. of toxin..	15	20	30	40	50
Number of units detected after forty-eight hours......	6	11	26	29	39
Number of units combined	9	9	10	11	11

* Lo dose of toxin, 0.18 cc. One cc. toxin neutralized by 5.5 units of antitoxin. Varying amounts of antitoxin in excess of 5.5 units added and after standing residual antitoxin titrated.

In the absence of the simpler animal test for the combining power of modified toxin, Glenny, Hopkins and Pope suggest the use of the flocculation test of Ramon. By this method it is easy to detect whether the combining power of toxin has altered during modification. Glenny and Okell have recently proposed the use of the symbol Lf comparable with Lr, Lo, for the amount of toxin equivalent to 1 unit of antitoxin, as determined by flocculation.

Table 6 shows the effect of exposing a toxin to various concentrations of formaldehyde at a temperature of 37° C. for twenty-four hours. The floccu-

TABLE 6.

SHOWING THE EFFECT ON A BATCH OF DIFFERENT TOXINS OF TREATMENT WITH VARYING CONCENTRATIONS OF FORMALDEHYD AT A TEMPERATURE OF 37 C. FOR TWENTY-FOUR HOURS (GLENNY, HOPKINS AND POPE)

Key Number	Percentage formaldehyde added. per cent.	Number of minimal reacting doses per cc.	Minimal lethal dose cc.	Lf, C.c.
J. 3488	Nil	500,000	0.002	0.11
Px 146	0.01	260,000	0.003	0.11
Px 147	0.03	150,000	0.01	0.11
Px 148	0.1	25,000	0.02	0.11
Px 149	0.2	5,000	0.05	0.12
Px 150	0.3	1,200	0.1	0.12
Px 151	0.4	50	2.0	0.12
Px 152	0.5	0	Over 5.0	0.12
Px 153	1.0	0	Over 5.0	0.12

lating doses recorded in the last column show that the total combining power of the toxin had not been greatly reduced, but that some loss had occurred. This could indicate that during the process of conversion of toxin into toxoid either destruction has occurred, or the affinity of the toxoid for antitoxin had been so greatly reduced that even the flocculating value had been affected. The latter is most probably true.

PERSONAL OBSERVATIONS

Several toxoid preparations were made by Banzhaf at the research laboratory according to the English method. These preparations of toxoid were used by Zingher for active immunization on an extensive scale among the school children of New York City. Tables 7 and 8 indicate in a striking way the results noted by him with two different preparations of toxoid.

(a) PREPARATION OF TOXOID USED FOR IMMUNIZATION

To each 1,000 cc. of toxin 1 cc. of formaldehyd was added. The bottles were shaken up and placed in the incubator, where they were allowed to remain undisturbed at a temperature of 38 C. for from four to five weeks. The preparations were then removed and tested for the minimum lethal dosage and the L +.

(*a*) Toxoid 437 used in the schools, enumerated in Table 7, was made from an old toxin which originally, two years ago, had a minimum lethal dosage of 0.001 cc. Before the addition of formaldehyd the toxin had a minimum lethal dosage of 0.01 cc. and an L + of 0.32 cc. After the addition of 0.1 per cent. formaldehyde and after remaining in the thermo tat for five weeks, the modified toxin was removed, and kept in the icebox until it was standardized and used for immunization.

The minimum lethal dosage of the toxoid was found to be 1.5 cc. (1 cc. killed a guinea-pig in thirteen days) and the L + 3.5 cc. The toxin had been reduced in toxicity by the action of formaldehyd and exposure to incubator temperature to the extent that 150 times the original amount of toxin represented one minimum lethal dosage. The combining power of the toxoid for antitoxin, however, had diminished much less, about ten times the amount of the original L + being represented by the new L +. The toxoid was diluted with salt solution, 1:20, 1:14 and 1:10, representing 5, 7.5 and 10 per cent. of the preparation.

The amino-acid nitrogen content of this toxoid, as determined by the van Slyke method, was 4.5 mg. for each 10 cc.

(*b*) Toxoid 377 was prepared from another toxin, which had a minimum lethal dosage of 0.01 cc. and an L + of 0.27 cc. One liter of the formaldehyd treated toxin, which had been kept in the incubator for four weeks and one liter which had been kept for five weeks, were mixed together and standardized. The new minimum lethal dose was approximately 1.0 cc. (1.0 cc. killed a guinea-pig in 4.5 days) and the L + was 5.0 cc. In contrast with Toxoid 437 this preparation had not lost as much in toxicity, yet in combining power as represented by the L + dose it was only about one-half as strong. The animo-acid nitrogen content of this toxoid was 5.1 mg. per 10 cc. These differences probably explain the difference in immunizing value noted between the two preparations.

(*b*) LOCAL AND CONSTITUTIONAL REACTIONS AFTER INJECTIONS OF TOXOID

The reactions following the injections of toxoid were rather mild when compared with those noted after injections of the old type 3 to 5 L + mixtures of toxin-antitoxin. The protein effect of the diluted toxoid was slightly more marked than that of the 0.1 L + mixtures of toxin-antitoxin. This was due to the dilution of the toxoid being from ten fold to twenty fold as compared with the thirty fold dilution of the toxin in the 0.1 L + toxin-antitoxin. The local toxic effect, however, of the diluted toxoid was decidedly less pronounced than after the 0.1 L + mixtures. This was due to the fact that each dose of toxoid represented only from one-twentieth to one-thirtieth of a minimum lethal dosage, as compared with the one-eighth minimum lethal dosage in each dose of the toxin-antitoxin.

(*c*) IMMUNITY RESULTS WITH TOXOID

Table 7 shows the results noted with Toxoid 437. It was diluted to 5 per cent. (1:20) for Group A, to 7.5 per cent. (1:14) for Group B and to 10 per cent. (1:10) for Group C. The table shows the results with two doses and with three doses of 1 c.c. each, given at intervals of from one to two weeks. Over 1,800 injected children were retested.

TABLE 7.

RESULTS OF ACTIVE IMMUNIZATION WITH DIPHTHERIA TOXOID (MODIFIED TOXIN) (ZINGHER)

Preparation No. 437. Tests by Banzhaf
Minimum lethal dosage of toxin two years ago, 0.001 c.c.
Before modification with 0.1 per cent. formaldehyde, minimum lethal dosage 0.01 c.c. L + 0.32 c.c.
After modification with 0.1 per cent, formaldehyde and after five weeks in thermostat, minimum lethal dosage 1.55 c.c. (150×) (Weaker). L + 3.5 c.c. (10×) (Weaker)
Nitrogen content by van Slyke method, 4.5 mg. per 10 c.c.

	Public School	Original Schick test		Diluted toxoid 1 cc.				Two doses toxoid			Three doses toxoid		
		Number tested	Per cent. positive	Dilution	Per cent. in dilution	Mos. after injection	Total retested	Number	Schick negative	Per cent. negative	Number	Schick negative	Per cent. negative
A.	103 M.*...	471	37.1	1:20	5.0	3	136	24	12	50.0	112	94	84.0
B.	14 M.....	686	18.5	1:14	7.5	3	125	25	20	80.0	100	98	98.0
	19 M.....	841	22.0	1:14	7.5	3	142	20	15	75.0	122	120	98.3
	83 M.....	625	17.3	1:14	7.5	3	81	14	14	100.0	67	66	98.5
	Total....	2,152	....	....	..	..	484	83	61	75.2	401	378	94.2
C.	15 M.....	714	25.6	1:10	10.0	3	145	11	20	55.0	134	141	95.0
	23 Bx.*...	600	24.8	1:10	10.0	3	98	15	11	73.3	83	77	92.7
	11 Bx.....	205	69.2	1:10	10.0	3	118	51	35	68.6	67	62	92.5
	32 Bx.....	174	30.0	1:10	10.0	3	29	3	2	66.6	26	24	92.3
	20 M.....	713	15.0	1:10	10.0	3	106	20	12	60.0	86	79	91.8
	3 Bx.....	741	24.4	1:10	10.0	3	132	27	21	77.7	105	94	90.4
	68 M.....	642	49.5	1:10	10.0	3	210	48	24	50.0	162	133	83.0
	85 M.....	725	11.1	1:10	10.0	3	43	16	12	75.0	27	24	81.5
	54 M.....	349	51.7	1:10	10.0	3	102	21	12	57.1	81	60	74.0
	61 M.....	1,253	25.5	1:10	10.0	3	274	33	18	54.5	241	176	73.0
	Total..	6,116	....	....	....	.	1,257	245	167	68.1	1,012	870	86.0

* M., Manhattan; Bx., Bronx.

Groups A and B have been added together. With two doses of the toxoid, sixty-one of eighty-three children, or 75.2 per cent., became immune;

with three doses, 378 of 401 children, or 94.2 per cent., were successfully immunized. In Group C the toxoid, although less diluted, gave somewhat poorer results. Of 245 children receiving two doses, 167 or 68.1 per cent., became immune and gave a negative Schick retest; of 1,012 children receiving three doses 870, or 86.0 per cent., were immunized and showd a negative reaction at the retest.

The results with this preparation of toxoid compare quite favorably with the immunity results formerly noted with many mixtures of toxin-antitoxin. As shown by Zingher in judging the immunizing value of a preparation of toxin-antitoxin, it is necessary to consider other factors besides the mixture itself. One of the most important of these is the previous exposure and slight immunization by contact of the injected children. It was found that the children of the poorer classes, showing a low percentage of positive reactions at the original test, when injected with the same preparation of toxin-antitoxin, gave a better antitoxin response than those of the more well-to-do, who had been kept fairly segregated and had had only slight exposure to repeated contact infection with the diphtheria bacillus. To a certain extent the same factors were found to hold true in the immunity results noted after injections of toxoid.

Table 8 gives the results with Toxoid 377. The immunity response was much poorer than with Toxoid 437. The dilution of the toxoid used in these schools was 5 per cent. (1:20). In other schools we injected the toxoid in a dilution of 7.5 per cent. (1:14), and these schools will be retested within a few months. With three doses of the 1:20 toxoid dilution, only 114 out of 245, or 46.5 per cent., were successfully immunized. The explanation given, that the actual combining value of this toxoid preparation was less than that of Toxoid 437, even though its toxicity for the guinea-pig was greater, probably explains these results.

TABLE 8.

RESULTS OF ACTIVE IMMUNIZATION WITH DIPHTHERIA TOXOID (MODIFIED TOXIN) ·(ZINGHER)

Preparation No. 377
Minimum lethal dosage of toxin two years ago, 0.001 c.c.
Before modification with 0.1 per cent. formaldehyd, minimum lethal dosage 0.01 c.c. L + 0.27 c.c.
After modification with 0.1 per cent. formaldehyd after five weeks in thermostat, minimum lethal dosage 1.00 c.c. (100×) (weaker). L + 5.0 c.c. (18.5 ×) (weaker)
Nitrogen content by van Slyke method, 5.1 mg. per 10 c.c.

	Original Schick Test					Two Doses Toxoid			Toxoid Three Doses		
Public School	Number tested	Per cent. positive	Toxoid dilution	Mos. after injection	Total retested	Number	Schick negative	Per cent. negative	Number	Schick negative	Per cent. negative
105 Manhattan........	296	32.0	1:20	3	71	00	00	00.0	71	36	50.7
51 Manhattan........	399	21.0	1:20	3	88	13	10	76.9	75	44	58.6
84 Manhattan........	330	31.2	1:20	3	61	19	6	31.5	42	21	50.0
37 Bronx...........	357	26.3	1:20	3	61	4	2	50.0	57	16	27.4
Total............	1,382	....	....	..	281	36	18	50.0	245	114	46.5

The retests in the schools shown in Tables 5 and 6 were given from 2.5 to 3 months after the injections. Zingher has found in his work with toxin-antitoxin that a second Schick test made a year later on the positive reactors will show that more than 50 per cent. will have become immune without their having received further injections of toxin-antitoxin. It is interesting to note in this connection that many of the children who are shown in Tables 7 and 8 as persistent positive Schick reactors, gave a much weaker reaction than in the original test. It is probable, therefore, that many will show a negative Schick reaction at a later retest.

COMMENT

The value of toxoid as an immunizing agent against diphtheria lies in its easy and simple preparation and standardization. It is also a very safe preparation to use.

The possibility that persons become sensitized to horse serum by the injections of toxin-antitoxin was indicated by the intradermal tests reported by Hooker. A repetition of Hooker's investigations by one of us gave somewhat different results, in that while those injected with toxin-antitoxin showed on retest an increased percentage of skin reactions to horse serum, yet this was much less than Hooker found. There is also great doubt as to the importance of the slight amount of sensitization. The knowledge of an immunization should not cause us to withhold antitoxin as persons have been repeatedly injected with antitoxin after previous injections of toxin-antitoxin, and no greater amount of clinical symptoms of sensitization was noted than after injections in persons who had not previously received toxin-antitoxin. However, to obviate even the slight possibility of danger, goat antitoxin is being substituted now for horse antitoxin in the preparation of toxin-antitoxin.

An observation made by Zingher in connection with the Schick retests after immunization with one-tenth L + mixtures of toxin-antitoxin is significant in connection with the question of sensitization after injections of these mixtures. He found that not only were from 80 to 95 per cent. of the injected children immunized, but also that over 50 per cent. of these children showed slight to marked pseudo-reactions in the Schick retest. With the 3 to 5 L + mixtures such a high percentage of pseudoreactions was never noted at the retests. The question comes up whether very small amounts of proteins contained in the one-tenth L + mixtures are not apt to be sensitized to a somewhat more intense degree than the larger amounts represented by the 3 to 5 L + mixture. Whatever the explanation may be, this sensitization after one-tenth L + mixtures has no clinical significance except so far as indicating the necessity for the sake of accuracy for always making a control test when the Schick retest is made to check up on the immunity results.

More important than the question of sensitization to horse serum after toxin-antitoxin injections, is the recent experience of White that a preparation of 1 L + of toxin-antitoxin after freezing became toxic, which has added further interest to the possibility of substitution of toxoid for toxin-antitoxin. Even though further investigation showed that the 0.1 L + preparation never became more toxic, yet a certain opposition, unreasonable though it may be, has developed among a portion of the lay population against the use of toxin-antitoxin which will be eliminated by the substitution of toxoid.

Further work no doubt remains to be done in perfecting toxoid as an immunizing agent against diphtheria. Similar work had to be carried out with toxin-antitoxin. But the results noted in this communication are sufficiently encouraging to continue the work on an increasing scale and to aim for a more perfect preparation. There is no immediate urgency to substitute toxin-antitoxin by toxoid. If, however, as is probable, a toxoid can be elaborated that will serve as a good immunizing agent, the time will not be far distant when such substitution will be found of advantage.

CONCLUSIONS

1. Both the new toxin-antitoxin and the toxoid preparations give good immunizing results with but slight local and constitutional reactions.

2. The toxoid has advantages in that there is no horse serum in it to cause sensitization, which happens to a slight degree with toxin-antitoxin. The substitution of goat antitoxin for horse antitoxin will obviate this difficulty and this change is now being made.

3. Such a toxoid would do away with the fear in the population created by the recent experience with frozen toxin-antitoxin.

4. A satisfactory toxoid preparation is probably easier to make and also more stable.

5. In making toxoid by the addition of formaldehyde to toxin, care has to be taken of the amount of formaldehyd added and the period of time the formaldehyd treated toxin is exposed to the temperature of the incubator. The amount of amino-acid nitrogen, as determined by the van Slyke method, has a definite bearing on the amount of formaldehyd that is necessary to be added. Many of the toxin preparations containing from 2.5 to 5 mg. of nitrogen per 10 c.c. will require the addition of 0.1 per cent. formaldehyde and the exposure in the incubator from four to five weeks.

6. Careful tests of the minimum lethal dosage, the L + and the flocculating value of the toxoid should be made in standardizing the preparation. The minimum lethal dosage represents the remaining trace of free acting toxin, the L + and the flocculating value, the combining power of the toxoid. The combining value of toxoid serves as a good index of its immunizing value. It is of advantage to have a slight excess of free toxin (from 1/15 to 1/20 minimum lethal dosage in each dose) in the toxoid.

7. Toxoid and toxin-antitoxin should be carefully studied and, if possible, improved, and the one that is shown to be the best adopted for general use.

For bibliography see the original article.

THE NEW MIXTURE (1/10 L +) OF TOXIN ANTITOXIN

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William H. Park, E. J. Banzhaf and Abraham Zingher

The extensive work in active immunization with diphtheria toxin-antitoxin, which was carried on during the past three years in the schools and baby health stations of this City, has enabled us to make a number of interesting observations and has led to certain conclusions as to the type of mixture of toxin-antitoxin that would have to be used before we could hope for a wide acceptance of this form of protection against diphtheria.

Until a short time ago we had used quite extensively mixtures of toxin-antitoxin that contained from 3 to 6 L + doses of toxin per cc. These mixtures were prepared from strong toxins which were only slightly diluted before the addition of the antitoxin. They contained relatively large amounts of different proteins which gave rise to the disagreeable local and constitutional symptoms associated with toxin-antitoxin immunization. Chief among these reacting substances is the autolyzed protein of the diphtheria bacillus, which is contained in the diphtheria toxin broth culture.

One of the important questions that had to be decided was whether a mixture of toxin-antitoxin with a large number of L + doses per cc. would give better immunity results than one with a smaller number of L + doses. We therefore used different mixtures in the schools, containing between 3 and 6 L + doses per cc. *We found that mixtures with the larger number of L + doses were not more efficient than those with the smaller number of L + doses, as long as the mixtures were equally toxic (underneutralized).* In fact we noted that mixtures with the *smaller* number of L + doses per cc. but showing greater toxicity for the guinea-pig, gave *better immunity results* than those with the larger number which were more neutralized.

On the basis of these data Dr. Park thought that much smaller amounts of toxin might be equally valuable and asked Dr. Banzhaf to prepare mixtures of toxin-antitoxin which contained only a fraction of an L + dose of toxin per cc. These mixtures were to be balanced, however, in such a way (see Fig. 1) that the toxic or underneutralized fraction of the toxin-antitoxin was similar to that of mixtures containing from 3 to 6 L + doses of toxin per cc., which had been found to give an active immunity in a large percentage of children. By diluting the toxin and thus greatly diminishing the reacting protein content of the mixture, it was hoped to eliminate one of the most objectionable features of diphtheria toxin-antitoxin immunization. Fig. 1 shows that in the new type mixture, 29/30 of the neutralized fraction of toxin-antitoxin has been eliminated by dilution, while the free* or toxic fraction has been retained in the same proportion as in an efficient old type (3 L +) mixture.

Preparation of the New Mixture

Several mixtures of toxin-antitoxin, containing from 1/20 to 1/2 L + doses of toxin per cc. were prepared, some of these being slightly more toxic than others. After these mixtures had been used in a number of institutions by Schroeder a standard mixture was adopted with a definite fraction of an

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*The words "free toxin" are used in this article to indicate the proportion of toxin in a mixture of toxin-antitoxin which is not neutralized by antitoxin.

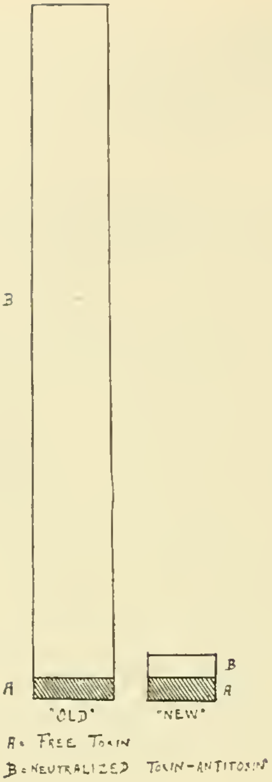

Fig. 1

Diagram of the composition of "old" and "new" toxin-antitoxin mixtures, each containing the same amount of free toxin. "Old" contains 3 L+ doses of toxin and 3.5 units of antitoxin per cc. "New" contains 0.1 L+ dose of toxin and 0.075 unit of antitoxin per cc. (Reprinted by courtesy of N. Y. State Journal of Medicine.)

L + dose of toxin per cc. This standard mixture was taken to be one containing 1/10 L + per cc. and representing about 4 M. L. D. of our toxin.

Park, Schroeder and Zingher reported in the *American Journal of Public Health* (January, 1923), from observations made by Dr. Schroeder in a few schools, that 90 per cent of 490 children became immune after three doses of a 1/10 L + mixture of toxin-antitoxin. Of 318 children injected with a 3 L + mixture, 92 per cent. became immune. Marked local reactions were noted in 22 per cent. with the old mixture and in none with the new type mixture.

The new mixture of toxin-antitoxin containing 1/10 L + per cc. is prepared by Dr. Banzhaf as follows: The antitoxin is added to the toxin in the proportion of 0.75 unit of antitoxin to one L + dose of toxin, the mixture allowed to stand for three to four hours and then diluted, so that each cc. represents 1/10 L + dose of toxin. The diluted mixture is subsequently passed through a Berkefeld filter, a sample of the mixture being removed before and after filtration to determine its toxicity in the guinea-pig. Six guinea-pigs are injected with each sample, two receiving 1 cc. each, two 3.0 cc., and two 5.0 cc. Daily observations are made on the loss of weight, local induration at the site of injection and late paralysis. If the mixture is found to be too toxic it is allowed to remain at ice-box temperature for one to three months. It is then tested in a similar way until the desired toxicity is found to be present. The preservative added to the mixture of toxin-antitoxin is 0.5% phenol. We have found that tricresol added as a preservative to toxin-antitoxin is too irritating locally, the children complaining of a severe burning sensation at the time of the injection.

STANDARD OF TOXICITY OF THE NEW MIXTURES

As a result of rapidly accumulating experience with mixtures of toxin-antitoxin of different degrees of toxicity, we have come to the conclusion that an *underneutralized mixture, of which* 5.0 *cc. causes acute death of the guinea-pig in five to six days,* 3.0 *cc. death in six to ten days, and* 1.0 *cc. paralysis in fifteen to eighteen days, and death in eighteen to twenty-five days is the best type of mixture to be used for active immunization.*

LOCAL AND CONSTITUTIONAL REACTIONS PRODUCED BY NEW AND OLD TYPE MIXTURES

The local and constitutional reactions obtained with mixtures of toxin-antitoxin are due to two different substances in the mixtures:

(A) In the old type mixtures, containing from 3-6 L + doses of toxin per cc., there was a considerable amount of autolyzed bacillary substance and other kinds of protein derived from the broth culture itself, which gave rise in sensitive individuals to the most marked forms of local and constitutional reactions. These reactions were of an anaphylactic type and generally occurred in hypersensitive individuals, who had given a negative-pseudo or positive-combined reaction. Both the local and constitutional reactions reached their height within twenty-four hours, gradually disappeared in two to three days, although they persisted at times for four to six days. The local reaction varied from an area of redness and induration the size of a 50-cent-piece to an extensive infiltration involving one-half or more of the arm. There was pain, tenderness and slight glandular enlargement in the axilla. Fever was present, the temperature ranging from 100-104° F. A few of the individuals showing simple positive Schick reactions also had slight local and constitutional disturbances, but the more marked reactions to the toxin-antitoxin were found only in those who had shown themselves to be hypersensitive to the proteins of the mixture by their negative-pseudo or positive-combined reactions.

(B) With the new type of mixture (1/10 L +) the local and constitutional effects due to the reacting protein were to a large extent eliminated, but the local effect of the larger amount of free toxin present in some of these preparations became more evident. We noticed, following the subcutaneous injections, especially with mixtures of which 1.0 cc. caused death of the guinea-pig in six to eight days and 3.0 cc. in three to five days, local reactions that were slow in appearing, but much more lasting than the protein reactions. These reactions were characterized by local induration and redness, which gradually faded into a brownish-red area of discoloration. The area had a dry, wrinkled, desquamating appearance and resembled closely on inspection a large positive Schick reaction. With one of these mixtures we even noticed in a few children a superficial necrosis of the skin. This local reaction was due to a large extent to the free toxic fraction of the mixture.

The majority of the mixtures of toxin-antitoxin used in the schools were less toxic and corresponded more to the degree of underneutralization given for a standard preparation. With such mixtures the local reactions were only slight and often absent. It was possible, in fact, to inject several hundred school children with these new type mixtures during a day's work without hearing subsequently of complaints about sore arms, or the necessity of the children remaining away from school for a day or two. Constitutional symptoms like fever, were practically absent. This was noticeable even after injecting the more toxic preparations, and stood out in striking contrast to the disagreeable effects seen with the 3-6 L + mixtures in 5-20 per cent. of school children.

Limits of Underneutralization

The rather severe local effects of some of the more toxic mixtures of toxin-antitoxin indicate the upper limit of toxicity within which we have to confine ourselves if we wish to avoid disturbing local reactions. If we take care, however, not to exceed this toxicity in the new mixtures and adhere more or less closely to the degree of underneutralization indicated as a suitable standard, we shall find in the 1/10 L + mixture of toxin-antitoxin an ideal preparation for the active immunization against diphtheria.

Stability of the New Mixture

Recent results obtained with the old type mixtures (3-5 L + per cc.) of toxin-antitoxin (Table 1) indicate that these mixtures do not lose to any extent their *immunizing* value even after standing for several months. Some of these mixtures had lost nearly all their *toxicity* on standing and appeared almost neutralized in the guinea-pig test. The slight excess of free toxin being changed into toxoid, *these favorable immunity results indicate that toxoid can give rise to antitoxin antibodies and that it is probably an important factor in active immunization.* We are giving three injections of diluted diphtheria toxoid at the present time to determine its value in producing an active immunity against diphtheria. A similar method has been suggested by Glenny and Hopkins.

TABLE 1.

RESULTS OBTAINED WITH FRESH MIXTURES AS COMPARED WITH OLDER MIXTURES OF TOXIN-ANTITOXIN CONTAINING FROM 3 TO 5 L + TOXIN PER CC.

Mixture	No. L+ in each CC.	No. M. L. D. in each CC.	Amount injected	Action on Guinea-Pig	Results in Children			
				Result	No. Doses T-A	Total No. Schick Retested	Schick Negative	Per Cent. Negative
44 Fresh	3	120	1.0 cc. 5.0 cc.	Acute death in 6 days. Acute death in 3 days.	3	1,080	1,047	97.0
44* Old	3	120	1.0 cc. 3.0 cc. 5.0 cc.	Slight local effect, death from pneumonia. Moderate local effect, death in 12 days. Moderate local effect, death in 9 days.	3	142	137	96.4
42 Fresh	5	200	0.5 cc. 1.0 cc. 5.0 cc.	Paralysis, death in 17-20 days. Paralysis, death in 17 days. Acute death in 3 days.	3	3,846	3,356	87.2
42* Old	5	200	1.0 cc. 2.0 cc. 5.0 cc.	No local effect, no paralysis, recovery. No local effect, no paralysis, recovery. Slight local effect.	3	364	361	99.1

*The mixtures were kept at ice box temperature for 7 months.

IMMUNOLOGICAL STUDIES ON TYPES OF DIPHTHERIA BACILLI

I. Agglutination Characteristics
II. Protective Value of the Standard Monovalent Antitoxin

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William H. Park, Anna W. Williams and Alice G. Mann

The subject of this communication would have been exhaustively investigated long ago if there had been practical evidence that a monovalent diphtheria antitoxin did not protect from diphtheria. It is true that in regard to *B. tetani* and *B. botulinus* laboratory investigations have given contradictory results as to the efficacy of a monovalent serum. These investigations have shown us: first, that tetanus bacilli, although they are divided into several types by their response to agglutinins, nevertheless, all produce toxins which are neutralized by a monovalent antitoxin; second, that the reverse is true of the bacilli causing bottulism, in so far as the production of toxin is concerned. Among the strains of *B. botulinus* there are at least two different types producing toxins which respond only to homologous antitoxins.

As to diphtheria bacilli, however, there are two series of observations which have been made concerning the degree of protection afforded by monovalent diphtheria antitoxin. These observations have seemed to give all-sufficient evidence that the toxins produced by the different strains of the diphtheria bacilli are alike, at least, in their affinity for antitoxin. The first is, that for the last twenty-five years, ever since diphtheria antitoxin has been used in practice, injections of a monovalent antitoxin in hundreds of thousands of persons known to be in contact with infection, have given practically complete protection for two weeks—the period during which antitoxin is known to remain in appreciable amounts in a human being after injection. The second series of observations is that routine virulence tests throughout the world have with few, if any, exceptions shown that a monovalent diphtheria antitoxin is able to protect animals against a dose of diphtheria culture fatal to other animals not given antitoxin.

If any doubt has existed in regard to the universal worth of a monovalent diphtheria antitoxin, it is one that has led to experimental investigation from time to time as to whether an antitoxic serum alone was as efficient as a combined antibacterial and antitoxic serum and whether from this standpoint a polyvalent serum might not be more advantageous. But the paper published by Havens in 1920 seemed to show that diphtheria bacilli were divided into two agglutinative groups and that each group produced a toxin that was quantitatively at least, a very different one from that produced by the other

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This article combines the results of portions of two investigations. The first portion was a part of our studies on the respiratory infections and was designed to show the relative values of the absorption-agglutination method and other serological tests in determining epidemic strains. The second portion was a part of a series of studies on the similarity of the toxins prodouced by the diphtheria bacilli and the adequacy of a monovalent antitoxin.

In the studies on the absorption-agglutination characteristics of the diphtheria bacilli, we were given valuable aid by technicians supplied by the Influenza Commission of the Metropolitan Life Insurance Company.

group, so that a monovalent serum for group I had no appreciable effect on animals injected with group II toxin unless the serum was given in enormous amounts. These findings, if correct, are of so much practical importance that reference will be made to them in some detail in connection with our own work.

I. Agglutinative Types of B. Diphtheria

A number of investigators have reported the obtaining of an agglutinating serum that differentiated between "true" and "pseudo" diphtheria bacilli (Schworer, Sander, Mason, but no one had described definite agglutinative types among the toxic strains until Durand published his observations in 1918. Durand announced that he had obtained four agglutinative types. In 1920 he published a further report in which he stated that on examining 252 strains by this test he found that they were divided as follows:

Type A contained	16	strains
Type B contained	8	strains
Type C contained	31	strains
Type D contained	76	strains
Type E contained	51	strains
Unclassified	71	strains

These types were strictly specific in that each absorbed only the respective agglutinins from the homologous serum.

Early in 1920 Dr. Durand visited our laboratory, demonstrated his methods, presented us with his type strains which he now designated with figures instead of letters and gave us the following facts in regard to their sugar fermentations:

	MALTOSE	DEXTRIN	GLYCEROL	GALACTOSE	SACCHAROSE
Type I—Americaine ("our No. 8")	+	+	−	−	−
Type II—Durand	−	−	−	−	−
Type III—Nodet	+	+	+	+	−
Type IV—Benjamin	+	+	+	+	−
Type V—Sirbeaux	+	+	+	+	−

All ferment dextrose and levulose.

Dr. Durand also gave figures to support the following observation: There was less paralysis and attendant mortality in cases of diphtheria treated with diphtheria serum produced by the inoculation of both toxin and bacilli; i.e., an antitoxic and "antibacterial" serum, than with serum produced by the use of toxin alone. As Durand used all of the type strains in making this bacterial serum he had no evidence as to the effect of a monovalent antibacterial serum.

Our own work on agglutination. We immediately began inoculating a series of horses with Durand's type strains and with our own strain of "No. 8," as well as with several strains from each of two apparent epidemics of diphtheria, one in an institution in New York City and the other in a village near the City.

The horses were inoculated intravenously every day for three day periods, with suspensions of cultures prepared as follows:

The bacteria were grown for eighteen hours on Loeffler's coagulated blood serum medium at 37° C. The growths were then scraped into 0.85 per cent. salt solution and transferred to tared Purdy tubes. They were sedimented in the centrifuge for twenty minutes and the supernatant liquid was removed. The weight of moist sediment was determined and the sediment was suspended in 10 cc. salt solution. The required weight of sediment (in suspension) was measured off and made up to 15 cc. for injection.

TABLE 1.

DIRECT AGGLUTINATION OF THE STRAINS OF B. DIPHTHERIAE USED FOR TOXIN-ANTITOXIN COMPARISON

STRAINS	HORSE SERUM TITER					
	No. 8	Nodet	Sirbeaux	Benjamin	11	3362
Type I. Our "no. 8"	1600	0	0	0	0	
Type I. Americaine	1600	0	0	0	0	
Type III. Nodet	0	1600	0	0	0	0
Type IV. Benjamin	0	0	0	800	800	
Type IV. 11	0	0	0	0	0	
Type V. Sirbeaux	0	0	400	0	0	
No. 3362	0	0	0	0	0	400

The first dose was 1 mgm.; each succeeding dose was doubled unless the condition of the horse contra-indicated it. After each three day period, the horse was allowed to rest for seven days. The first three doses of the bacillus emulsion were heated at 55° C. for one hour before inoculation. Later, living cultures were inoculated. From four to six series of inoculations were given each horse.

In table 1 are given only the strains which were used by us to test the protective action of our monovalent serum.

With the serums obtained, we corroborated fully the work of Durand showing that there are at least five agglutinative types of diphtheria bacilli.

Havens kindly sent us four strains of his group II and we found that none of them agglutinated with our "No. 8" serum.

Havens did not mention Durand's work in his article. He reported the results of his own study of 206 strains. These were from acute cases, convalescents and carriers. All varieties of morphologic cultures were tested but he says that "morphology bears no observed relation to agglutinating properties." A certain strain was chosen to inoculate rabbits for the production of agglutinating serum. Havens does not give the dosage or the course of injections. He simply states that the injections were made intravenously in increasing amounts, and that an agglutinating serum was obtained with a titer of 4860 for the homologous strain. Of the series of 206 strains, 169 agglutinated in this serum to the same titer as the homologous strain, while 37 failed to agglutinate in any dilution. One strain of this second group was chosen to inoculate a rabbit for the production of agglutinating serum. This serum gave a titer of 4800 or higher. The other 36 strains of this group agglutinated to the same titer. He concludes as follows:

Judged by the evidence furnished by the agglutination test there are two biologic groups of the diphtheria bacillus. No evidence of cross agglutination was found among the members of this series [obtained in Iowa City].

All members of the series fell into one of the two groups with no differences observed in the degree of agglutinability. As judged by the results of agglutination, two groups exist which include all strains of the diphtheria bacillus.

Havens said that the virulence in animals was approximately the same in the two groups.

When we consider the results of Havens in connection with Durand's and those of our own, it seems fair to infer that in the comparatively small city from which he drew his material, only two types of bacilli were present.

There can be no doubt whatsoever that in all large communities there are many types and at least several dominant ones.

II. Protective Action of Diphtheria Antitoxin (Standard No. 8), Against Different Agglutinating Types of B. Diphtheriae

The question of the protection results is so important that we quote Havens' experiments in detail. In attempting to show "the protective properties of diphtheria antitoxin with reference to biologic groups," he reports first the following tests with various cultures and diphtheria antitoxin No. 8.

HAVENS' TESTS

CULTURE	GROUP	AMOUNT	UNITS ANTITOXIN	TYPE ANTITOXIN	RESULT
		cc.			
210	I	1	None		Died, 48 hours
210	I	1	100	I	Lived
221	II	1	None		Died, 36 hours
221	II	1	100	I	Died, 36 hours
141	II	1	None		Died, 48 hours
141	II	1	100	I	Died, 48 hours

The results of these tests of Havens indicate that group I antitoxin has no neutralizing effect upon the toxins produced in the animals by group II cultures. Similar results followed when he injected cultures intracutaneously. Six strains of group I bacilli were inoculated into guinea pigs that had been injected with 100 units of group I antitoxin. No cutaneous lesions were produced. Eighteen strains of group II bacilli were inoculated into guinea pigs that had received 100 units of group I antitoxin. The reactions in 15 were identical with the controls, while those in three were slightly less than the controls.

Before discussing Havens' results with toxin, we give in table 2 our findings with cultures of at least five different agglutinating types including three of Havens' "group II" strains. The cultures were heavy eighteen hour growths in veal broth.

TABLE 2

LIFE-SAVING EFFECTS OF TYPE I ANTITOXIN IN ANIMALS INJECTED WITH BACILLI OF VARIOUS TYPES

TYPE	WEIGHT OF GUINEA PIG	AMOUNT OF CULTURE	UNITS OF ANTITOXIN NO. 8	RESULT
	grams	*cc.*		
Type I. (Our no. 8, New York City)	248	2.0	None	Died 3 days
	230	3.0	None	Died 2 days
	355	3.0	None	Died 2 days
	230	3.0	0.2	Died 2 days
	230	3.0	0.5	Died 2 days
	360	3.0	1.0	Remained well
	355	3.0	2.0	Remained well
Type III. (Durand's Nodet, France)	225	0.2	None	Died 2 days
	225	0.2	0.5	Remained well
Type V. (Durand's Sirbeaux, France)	235	0.2	None	Died 9 days
	400	0.75	None	Died 4 days
	235	0.2	0.2	Died 8 days
	405	0.75	1.5	Remained well
Type IV. (No. 11, New York City)	242	2.0	None	Died 3 days
	245	2.0	0.5	Remained well
Type IV. (Durand's Benjamin, France)	240	0.05	None	Died 2 days
	235	0.1	0.2	Died 6 days
	235	0.05	0.05	Remained well
No. 3362, unclassified (New York City)	245	0.2	None	Died 3 days
	255	0.2	0.2	Died 4 days
	255	0.02	0.5	Remained well
B, unclassified (Near New York City)	235	0.1	None	Died 6 days
	245	0.1	0.2	Remained well
No. 151 (Havens' group II, Iowa City)	237	1.0	None	Died 3 days
	262	1.0	None	Died 2 days
	255	1.0	0.2	Remained well
	245	1.0	0.5	Remained well
No. 184 (Havens' group II, Iowa City)	225	0.2	None	Died 2 days
	232	0.2	None	Died 2 days
	245	0.2	0.2	Died 6 days
	230	0.2	0.5	Remained well
No. 8347 (Havens' group II, Iowa City)	217	0.1	None	Died 10 days
	440	0.3	None	Died 2 days
	235	0.3	0.5	Died 3 days
	225	0.3	1.0	Died 3 days
	215	0.3	5.0	Remained well
	255	0.3	5.0	Remained well

The results given in the table do not show any striking difference in the protecting value of the type I antitoxin, when used in animals inoculated with a type I strain or with strains of other types. All who have worked extensively with living cultures have discovered that the results are much more irregular than when toxins are used. The cultures vary from day to day, according to the culture medium used, in the abundance of their growth and in the vigor and apparent toxicity of the bacilli; a much larger number of units of antitoxin is required to protect against a fatal dose of living bacilli than against a fatal dose of toxin. There is nothing in our results to indicate to us that there is any marked difference in the pathogenic power of the different types of bacilli.

The last three cultures are of unusual interest since they were sent us by Havens as examples of his Group II strains. All produced toxin of moderate strength when grown in bouillon. It is to be noted that our antitoxin produced by type I No. 8 bacillus was capable of protecting the animal which had received a subcutaneous injection of an amount of culture which produced death in the control animal within two days. Indeed, the type I antitoxin appears to be as efficient against two of the cultures sent by Havens as against strain "No. 8." Although with the third culture a considerably larger quantity of antitoxin was required, it is noted below in table 3 that the toxin produced by this culture was neutralized by the usual amount of antitoxin.

The experiments of Havens showing the neutralizing results of mixtures of his group I antitoxin and group II toxin are given in the following tabulation:

HAVENS' TESTS

STRAIN NUMBER	TOXIN GROUP	MINIMAL LETHAL DOSES	GROUP I ANTITOXIN UNITS	RESULT
53	II	2	10	Died, 72 hours
53	II	2	—	Died, 72 hours
74	II	2	5	Died, 48 hours
74	II	2	20	Lived, marked virulence
74	II	2	25	Lived, oedema
74	II	2	50	Lived, no lesions
34	I	2	5	Remained well
34	I	2		Died in 48 hours

The group II toxin required far larger amounts of group I antitoxin to neutralize it than did the group I toxin.

NEUTRALIZING EXPERIMENTS WITH MIXTURES OF GROUP II ANTITOXIN AND GROUP I TOXIN

STRAIN NUMBER	TOXIN GROUP	MINIMAL LETHAL DOSES	GROUP II ANTITOXIN UNITS	RESULT
34	I	1	5	Died, 96 hours
34	I	1		Died, 96 hours

As seen in the above tabulation, one minimal lethal dose of group I toxin was not neutralized by five units of group II antitoxin.

The fact that in Havens' tests very large amounts of group I antitoxin usually neutralized 2 minimal lethal doses of group II toxin, indicates that the group I antitoxin contained the same neutralizing substances for group II toxin as for group I toxin but that there were quantitative differences in the proportion of the different antitoxic substances.

TABLE 3.
TOXIN RESULTS

TOXIN		WEIGHT OF GUINEA PIG	NUMBER OF MINIMAL LETHAL DOSES	NO. 8 ANTITOXIN	RESULT
From culture	Type				
		grams			
No. 8	I	250	1.0	0.02	Lived
Durand	II	230-260	1.0	0.02	Lived
Nodet	III	230-260	1.5	0.02	Lived
Benjamin	IV	230-260	2.0	0.02	Lived
No. 11	IV	230-260	1.0	0.02	Lived
3362	Unclassified	230-260	2.0	0.02	Lived
151	Havens, group II	230-260	1.0	0.02	Lived
8347	Havens, group II	230-260	1.5	0.1	Lived
8347	Havens, group II	230-260	100.0	10.00	Lived

Havens stated "the results may throw some light on those cases of diphtheria which are not benefited except by large amounts of antitoxin." He alluded also to the death rate remaining at 10 per cent in cases of diphtheria. He suggested adding his group II toxin to horse injections, to the Schick test toxin and also to toxin-antitoxin mixtures.

Table 3 gives the results which we obtained with neutralizing mixtures of our own antitoxin No. 8 and the types of toxin indicated.

It is evident from this table that with the strains obtained from New York, from France and from Iowa no such difference has been discovered, as Havens believes he found. It would take repeated experiments and many animals to prove whether or not there is any minor difference either quantitatively or qualitatively between these toxins produced by the different agglutinative types.

CONCLUSIONS

The group of diphtheria bacilli contain strains belonging to several agglutinative types

The toxins formed by these different types are, however, qualitatively alike and, from the practical standpoint, quantitatively so. Whether or not there are slight quantitative differences, further studies will be necessary to show.

Strong toxin from any diphtheria bacillus strain is suitable for the Schick test and for immunization of man or animal.

A monovalent antitoxic serum is suitable for protective and curative measures against all diphtheria strains.

For bibliography see the original article.

HUMAN HYPERSENSITIVENESS TO WHOLE HORSE SERUM OR SERUM GLOBULINS FOLLOWING DIPHTHERIA TOXIN-ANTITOXIN INJECTIONS—ITS IMPORTANCE.

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William H. Park and May C. Schroeder

This preliminary communication is made at this time because of the interest aroused by Hooker's communication at the December meeting of the Society of American Bacteriologists.

He observed that a considerable percentage of adults who had some months previously received three injections of diphtheria toxin-antitoxin (3 L plus preparation) developed a skin sensitiveness to horse serum.

Two series of observations are here presented, one on children, the other on adults which confirm Hooker's conclusion, that a specific skin hypersensitiveness may be induced, in a moderate percentage of children and adults by the injection of the minute amount of horse serum globulins contained in the toxin-antitoxin injections. The results of tests on untreated persons and on those who had received larger amounts of serum globulins are also given as a comparison. As the antitoxin globulin solution is universally used to supply the antitoxin in the toxin-antitoxin, the globulin solution is used as the testing substances in most cases. Where the whole serum was used as a control the results were almost identical.

Tables 1 and 2 give the results in one series of tests on two groups of children, the individuals in one of which had and in one of which had not received, so far as is known, injections of toxin-antitoxin. It will be noted that about one-half of the children in each group were injected intracutaneously with 0.02 cc. of a 1:10 dilution of horse serum, the other half receiving

TABLE 1.

REACTIONS FROM INTRACUTANEOUS INJECTION OF HORSE SERUM OR HORSE SERUM GLOBULINS IN ONE HUNDRED AND SIXTEEN CHILDREN WHO HAD PREVIOUSLY RECEIVED THREE INJECTIONS OF TOXIN-ANTITOXIN. OBSERVATIONS MADE FIFTEEN TO TWENTY MINUTES AFTER THE INJECTIONS

MATERIAL INJECTED	AMOUNT	WHEAL AND AREOLA		WHEAL ONLY		NO REACTION	
		Number	Per cent.	Number	Per cent.	Number	Per cent.
	cc.						
Solution of antoxic globulins, 1:10 dilution	0.02	17	23	33	44.5	24	32.5
Horse serum, 1:10 dilution	0.02	15	35.7	19	45.3	8	19
Total		32	27.5	52	45	32	27.5

TABLE 2.

CONTROL OBSERVATIONS ON NINETY CHILDREN WHO SO FAR AS KNOWN HAD NOT RECEIVED PREVIOUS INJECTIONS

MATERIAL INJECTED	AMOUNT	WHEAL AND AREOLA		WHEAL ONLY		NO REACTION	
		Number	Per cent.	Number	Per cent.	Number	Per cent.
	cc.						
Globulin solution, 1:10 dilution.....	0.02	9	20	13	29	23	51
Normal horse serum, 1:10 dilution..	0.02	1	2	22	49	22	49
Total.........................		10	11	35	39	45	50

the same amount of antitoxic globulin solution. The total amount of globulin in the solution was about double that contained in the same quantity of unrefined serum. The children who had received toxin-antitoxin had been injected with a mixture containing 0.1 L plus dose of toxin. This contains but one thirtieth of the amount of serum globulins as compared with the older form of mixture which Hooker had employed. The amount of horse serum globulin in each dose (1 c.c.) of the new mixture is only about 0.0002 of that in 1 cc. of normal horse serum.

Tables 1 and 2 show that the children who had received three injections of toxin-antitoxin one year previously exhibited a much higher percentage of reactors than the untreated children. This is especially true of those who were tested by the horse serum injections. It is possible, but not probable, that one or two of the control children who reacted to the globulin had been given toxin-antitoxin before they entered the institution. While the persistence of the wheal probably indicates a trace of sensitiveness in the skin still it seems desirable to limit the term hypersensitiveness to those in which an areola develops.

TABLE 3.

OBSERVATIONS ON THIRTY ADULTS WHO HAD PREVIOUSLY RECEIVED THREE TOXIN-ANTITOXIN INJECTIONS OF A 3 L PLUS PREPARATION

MATERIAL INJECTED	AMOUNT	WHEAL AND AREOLA		WHEAL ONLY		NO REACTION	
		ber Num-	Per cent.	Number	Per cent.	Number	Per cent.
	cc.						
Solution of antitoxic globulins, 1:10 dilution ...	0.02	10	33	17	57	3	10

TABLE 4.

CONTROL OBSERVATIONS ON ONE HUNDRED AND EIGHTY-SEVEN ADULTS WHO HAD NOT RECEIVED INJECTIONS OF TOXIN-ANTITOXIN NOR AS FAR AS KNOWN OF ANY SERUM

MATERIAL INJECTED	AMOUNT	WHEAL AND AREOLA		WHEAL ONLY		NO REACTION	
		Number	Per cent.	Number	Per cent.	Number	Per cent.
	cc.						
Globulin solution, 1:10 dilution ...	0.02	36	19	103	55	48	26

The second series of observations was made upon adult inmates of an institution for the care of the insane. In this second series we are more certain of the fact that the control cases had never received toxin-antitoxin than we were in the first series. The results in the adults are similar to those in the children except that there was a higher percentage of reactors among the untreated persons. In no one of these cases was the areola more than 2 inches in diameter.

As an additional control a series of observations was made on sixteen children who, because of positive Schick tests, received on their admission to the Willard Parker Hospital prophylactic doses of 1500 to 3000 units of antitoxic globulin solution. The results of the intracutaneous injections of horse serum in these children are given in table 5.

TABLE 5.

THE FOLLOWING PATIENTS HAD POSITIVE SCHICK REACTIONS ON ADMISSION AND WERE GIVEN PROTECTIVE INOCULATIONS OF ANTITOXIC GLOBULINS AND TESTED FOR SENSITIVENESS TO HORSE SERUM AT VARYING INTERVALS AFTER THEIR PROPHYLACTIC DOSES OF ANTITOXIN

CASE NUMBER	AGE	UNITS ANTITOXIN	DATE OF ADMINISTRATION	DATE OF TEST	0.1 CC. OF 1:10 HORSE SERUM	0.02 CC. OF 1:10 HORSE SERUM
1	10	2000	11/28	12/19	Small areola	Small areola
2	7	1500	11/30	12/19	Small areola	Small areola
3	2	1500	12/5	12/19	No change	No change
4	3½	2000	12/20	1/15	No change	No change
5	6	1500	11/23	12/19	Negative	Negative
6	2	1500	11/28	12/19	No change	No change
7	6	3000	12/21	1/15	Areola	Areola (small)
8	8½	2000	11/22	12/19	Marked areola	Marked areola
9	2½	2000	11/16	12/23	Negative	Negative
10	6	2000	12/15	1/15	Marked areola	Marked areola
11	4	2000	11/20	12/23	Slight areola	Wheal size of dime, slight areola, no pseudopods
12	6	5000	11/10	12/23	Marked areola, irregular, no wheal	Marked areola, irregular, no wheal
13	14	2000	12/6	12/19	Areola	Slight areola
14	13	5000	11/19	1/14	No change	No change
15	8	2000	12/4	12/19	Marked areola	Moderately positive
16	16	2000	12/29	1/15	Areola	Areola

In this series 62.6 per cent. of the cases showed evidences of definite skin hypersensitiveness by the development of both a wheal and an areola. This contrasts with 27 per cent. and 33 per cent. in those who had been treated with toxin-antitoxin. The reactions in six were moderate. The areola in these cases ranged from 1 to 2 inches in diameter. In the other four the reaction was more intense. In one the areola extended nearly around the arm.

These observations on these three series of cases, although they are made on a comparatively small number of persons, indicate clearly that a moderate proportion of those who have been injected with toxin-antitoxin develop skin hypersensitiveness to horse serum globulins, but that an injection of 0.5 cc. of serum or a solution of serum globulins causes this change in a much greater proportion of persons and produces in them a greater effect.

It is fair to conclude, therefore, that Hooker is correct in his conclusion that toxin-antitoxin contains enough serum globulin to sensitize moderately a certain proportion of those receiving three injections. This leads up to the question as to whether such a hypersensitive state as shown by the skin reaction indicates a peril to the persons showing it if later serum injections become necessary.

Some ten years ago I felt it wise to give an intramuscular injection of 5000 units of antitoxin to each of thirty children who had received 1000 units or more of antitoxin about four weeks previously. The majority showed within a few minutes following an intracutaneous injection of 0.04 cc. of serum globulin solution a more or less extensive wheal and areola. Many of these developed within a few minutes a rise of temperature and a local or generalized rash. In the most extreme reactions the temperature rose five degrees but within four hours it had returned to normal. This child never seemed sick. None of the thirty children showed any symptoms that were in the slightest degree alarming.

The practice of the Health Department has been from the first introduction of antitoxin to give it to patients suffering from diphtheria without regard to the presence of the skin reaction. So long as there was no history of asthma or objective signs of status lymphaticus the serum injections were given without fear of the sensitizing effects of previous injections. The results of the injections in the thirty cases just mentioned confirmed us in our opinion that at least a subcutaneous or an intramuscular injection of whole serum or of antitoxin globulin solution could be given properly in those requiring it without being guided by the reaction of the test for skin hypersensitiveness. We have continued this practice up to the present time and have had no serious accidents.

The gradual introduction of the intravenous method of giving the antitoxin globulin solution in severe cases of diphtheria and of whole serum in cases of pneumonia have made us consider very carefully whether it was wise to disregard the cutaneous test in the treatment of these cases. Recently several interesting observations have been made which bear on this matter. A child, J.P., tested intracutaneously developed after fifteen minutes a slight wheal with a surrounding area of erythema. Twelve thousand units of antitoxin were given intravenously. The child promptly became cyanotic, sat up and fought for air. Simultaneously a large wheal and an enlarged areola with definite long pseudopods developed at the site of the previous intracutaneous injection. Ten minims of a 1:1000 solution of adrenalin were injected intramuscularly and the symptoms quickly subsided. There was no chill. The temperature rose two degrees during the next four hours and fell by lysis. The result in this case might seem to indicate that even a very moderate intracutaneous reaction should act as a warning but the next case throws doubt on this reasoning. A few days afterwards one of the staff was found to have diphtheria. When tested by an intracutaneous injection he showed no reaction and 10,000 units of antitoxin were injected intravenously. Almost immediately he developed a short hacking cough with difficulty in breathing. The skin seemed puffy, very red and somewhat purplish. These symptoms lasted about five minutes and then subsided abruptly. The patient sank back white and weak. Forty minutes later there was a chill that lasted about ten minutes, the temperature rose to 102°.

During the past two weeks intravenous injections of antitoxin have been given to twelve small children, who had just before that treatment developed after an intracutaneous test small wheals with an areola of about ½ to 1¾ inch in diameter. One of these developed a moderate chill and three a slight chill. Intravenous injections were also given to six developing no reaction. One of these developed a slight chill.

These results allow us to draw certain tentative conclusions. Persons who do not possess skin hypersensitiveness may occasionally develop serious symptoms after an intravenous and even after an intramuscular injection of horse serum but they are less liable to do so than those who have the skin reaction.

Cases which show a moderate reaction after an intracutaneous injection as shown by an areola of one half to two inches may or may not show an accelerated reaction to a therapeutic dose of serum. The reaction is not as a rule marked and almost never dangerous.

Cases which show a marked skin hypersensitiveness are apt to develop within a few minutes a marked serum reaction upon the injection of a therapeutic dose of serum. The reaction from a subcutaneous or an intramuscular injection is not, however, at all likely to be a dangerous one and can be readily controlled. The reaction from an intravenous injection is also not dangerous in the great majority of cases, if the antitoxic globulin solution is given very slowly and is stopped at the first appearance of symptoms. I believe we are justified without waiting for the result of an intracutaneous test in giving an immediate intravenous injection in every severe case of diphtheria unless we see evidences of status lymphaticus or obtain a history of asthma.

Although Hooker's findings are corroborated to a degree by the observations recorded in this paper it should be emphasized again that the sensitization is not as marked as that developing after larger dose of antitoxin for passive immunization. Even in the latter cases as indicated by the personal experiences noted above we need have no especial fear of reinjecting antitoxin or serum. It would seem, therefore, that the lesser sensitization given by toxin-antitoxin was in all probability a nearly negligible factor.

As this report is preliminary in character there seems no need to refer to such analyses of the results of second injections of antitoxin as have been published. The acceleration of the reaction after second injections has been observed by many and this has been studied especially by Von Pirquet and Schick. In one or two instances a fatal result has been attributed to the fact that a previous injection had been given. Notwithstanding this, there remains the fact that practically all of the few deaths following the injection of antitoxin have followed the first injection.

The suggestion of Hooker that a goat antitoxin be available has been made before for passive immunization but not as a substitute for horse serum in toxin-antitoxin. The fact that such an antitoxin is not available is evidence in itself that the experiences with second injections has never been sufficient to raise the fears of physicians and therefore, a demand for such an antitoxin. Its preparation is perfectly feasible in the small amounts that would be required and I have already started its preparation. Not only will there be some advantage in preventing the moderate amount of sensitization which results from the toxin-antitoxin injections, but the removal from the minds of physicians of any fear of this change will be of importance. Further observations on the apparent sensitization are in progress and these experiences will be presented at a later date.

ANTITOXIN IN DIPHTHERIA—A COMPARATIVE STUDY OF THE USUAL METHODS OF ADMINISTRATION WITH THE INTRAPERITONEAL METHOD*

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E. S. Platou

Ever since the introduction of antitoxin as a specific agent in the treatment of diphtheria, there has been considerable discussion as to the most suitable means of administering it. The methods now in vogue, namely, the subcutaneous, the intramuscular, and the intravenous are used alone or in combination, depending on the severity of the case. Another route not previously employed for this purpose but commonly used in the giving of fluids to infants, and more recently even of whole blood, is the intraperitoneal route.

It is the purpose of this article to set forth the advantages and disadvantages of the respective methods. To do this it will be necessary to consider the following factors: (1) the relative technical difficulties involved; (2) the reaction, local and general; and (3) the rapidity of absorption.

Technique of Administration. It is conceded that the technique of subcutaneous and intramuscular injections is simple. The details are too well known to need repetition. One point should be emphasized, however. In the intramuscular injection it is important to insert the needle deeply into the muscles, otherwise a preparation thought to be in the muscles may have reached only the subcutaneous tissues.

Intravenous administration of antitoxin unfortunately is more difficult, and particularly so at the ages when diphtheria is most common. No doubt the skill of the operator modifies this factor to a certain degree but even in expert hands it may be a real problem. It was this inability to find suitable veins in infants with laryngeal and toxic diphtheria, where rapid absorption is so essential, that first prompted a search for another method—one which would afford simplicity of technique as well as rapidity of absorption.

The absorptive power of the peritoneum previously shown by many observers suggested this area as a possible route for antitoxin and since the safety of the procedure had been attested to by many, it remained only to be demonstrated that the serum itself was non-irritating and that its absorpion would warrant the use of the method in selected cases.

The technique of intraperitoneal injection is, of course, simple and has been well described by Blackfan: "The patient lies in the recumbent posture with the movements of arms and legs restricted by a restraining jacket. The most favorable site for the introduction of the needle is through the linea alba just below the umbilicus. The skin is prepared after the usual surgical method, with iodin and alcohol, and the area may be rendered anesthetic with ethyl chlorid. The solution is introduced by gravity from an infusion bottle. When the patient is restrained in the proper position and the site for the injection prepared, the skin and subcutaneous tissues are picked up between the thumb and the index finger and the needle, pointing upward, is inserted at an oblique angle. After it has penetrated the peritoneum the fluid is allowed to flow into the peritoneal cavity."

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*From the Willard Parker Hospital and the Research Laboratory.

Reaction. The local and constitutional symptoms following any method will of course be influenced by the type of the preparation of antitoxin used. This is well illustrated by the figures of Park, showing the advantage of refined antitoxin (antitoxic globulin solution) in 50 treated cases of diphtheria.

TABLE 1.
(PARK)

	Children treated with the whole serum. Per cent.	Children who were treated with the antitoxin globulins. Per cent.
Marked constitutional symptoms accompanied by a severe and persistent rash...........	28	0
Moderate constitutional symptoms accompanied by a well-developed erythema or urticaria	18	4
Very slight constitutional disturbance accompanied by a more or less general urticaria or erythema....................	20	8
No appreciable constitutional disturbance but more or less general urticaria or erythema	4	34
No appreciable deleterious after-effects whatever	30	54

	Duration of Rashes								Totals
Days	1	2	3	4	5	6	7	8	
Antitoxic globulin cases	5	7	5	2	3	—	1	—	23
Whole serum cases	1	4	10	1	10	3	2	5	36

In making this study, therefore, the qualitative factor was minimized by the use in all cases of the refined product of the Department of Health Laboratories, City of New York.

After subcutaneous and intramuscular injections there is usually more or less localized tenderness which lasts about two days. A small number of patients may show mild latent reactions associated with urticaria but ordinarily there are no appreciable constitutional symptoms.

The intravenous method causes, of course, no local discomfort, providing there is no escape of the serum into the surrounding tissue. The important consideration in giving it intravenously then is the degree of constitutional reaction. This was formerly a serious hazard but has been diminished to such a degree that today the method is used frequently on our diphtheria service with only occasional severe reactions—a fact directly attributable to the improvement in the product of the laboratory. In spite of careful precautions, however, there is a certain number of cases in which chills, hyperpyrexia and even convulsions occur. In the child already very sick with diphtheria, the question arises as to the wisdom of adding the possibility of such deleterious effects. Of course in a large number of cases the advantage of the immediate presence of antitoxin in the blood unquestionably overbalances not only this danger but also the harm which may ensue from waiting for absorption from the muscles or skin.

The intraperitoneal method, first tried out on rabbits, has, up to date, been given in twelve clinical cases. The patients varied in age from five months to fourteen years (the older ones being used to facilitate the collection of blood specimens). In none of these patients was there any evidence of local discomfort. One child developed slight urticaria on the seventh day but none showed any constitutional symptoms. The greatest temperature rise in any case was .8° F. In four there was no change, whereas in two an actual drop occurred. The average rise for twelve cases was .3° F.

Although the number of patients is too limited to make any accurate statement concerning general reactions, the indication is that these approximate the subcutaneous and intramuscular rather than the intravenous.

Absorption. It is this factor which after all is of first importance, not only in advanced cases, but also in those defined as mild because of the limited involvement. A small membrane not infrequently proves a false prognostic criterion in predicting subsequent paralyses.

Method of Estimation. To compare any one method with another from the standpoint of absorption, it is essential that we have an accurate measure. This is furnished by the quantitative antitoxin determination, according to Hygienic Laboratory Standards, on blood taken before and at stated intervals after injection.

Serum from the blood of an animal or man to be tested is mixed with a toxin of known potency and injected into a guinea pig. The quantity of serum is varied according to the amount of antitoxin to be tested for and retests are performed until it is demonstrated that a definite quantity of the serum protects against the test dose of toxin. From this is computed the amount of antitoxin per c.c. of serum.

Natural Antitoxin. An individual to be immune to diphtheria must have at least 0.03 units of antitoxin per cc. present in the blood. Theoretically of course a person contracting diphtheria is Schick positive and possesses no antitoxin. It has been demonstrated, however, that a considerable percentage of persons have developed some antitoxin by the end of the second or third day following invasion of the body by the infecting organisms.

With the view of determining the extent of the presence of antitoxin as well as avoiding any error in regard to the receipt of it before admission to the hospital, tests were carried out on fifteen initial bleedings. The results are given in Table 2.

TABLE 2.

AMOUNT OF ANTITOXIN PER CC. OF BLOOD SERUM PRESENT IN FIFTEEN CASES WITH DIPHTHERIA OF VARIOUS TYPES BEFORE INJECTION

Case No. 5.	None.	Case No. 14.	0.04 units.
Case No. 6.	None (toxic).	Case No. 15.	0.07 units.
Case No. 7.	0.04 units.	Case No. 16.	None.
Case No. 8.	0.04 units.	Case No. 17.	0.12 units.
Case No. 9.	0.07 units.	Case No. 18.	0.07 units.
Case No. 10.	None (toxic).	Case No. 19.	0.125 units.
Case No. 11.	0.06 units.	Case No. 21.	None (toxic).
Case No. 13.	0.09 units.		

All types are represented among this group of fifteen, such as carriers, nasal diphtheria and the most severe, neglected forms. Although the figures indicate an appreciable activity on the part of some of these individuals to make antitoxin, the quantities found do not bear out the statements of relatively large amounts occasionally reported. It is possible that some of the latter may be explained by unreliable histories concerning the injection of antitoxin previously.

Rapidity of absorption. *Intravenous.* The time element in this method is of course eliminated.

Table 3 shows the amount of antitoxin per cc. of blood serum present at different intervals after intravenous administration of 10,000 units.

TABLE 3.

(AFTER PARK & WILLIAMS)

5 minutes after	13.6 units
24 hours after	11.0 units
3 days after	8.9 units
5 days after	7.4 units
7 days after	6.4 units

(See chart also.)

(For purposes of comparison these figures have been corrected according to weight and represent an average of four cases. Throughout this study a standard weight of thirty-five pounds will be maintained.)

Subcutaneous. The figures obtained after subcutaneous injection of 10,000 units are:

TABLE 4.

(AFTER PARK & WILLIAMS)

24 hours after	2.0 units
3 days after	4.4 units
4 days after	3.8 units
5 days after	4.1 units
7 days after	3.4 units

(These figures have also been corrected for weight.)

Intramuscular and Intraperitoneal. The preliminary tests on rabbits showed a rather striking difference between these two methods.

TABLE 5

(COMPARATIVE ABSORPTION IN RABBITS)

Intramuscular	(10,000 units)	Intraperitoneal
2 units per cc. blood serum	2 hours	19 units per cc.
3 units per cc. blood serum	4 hours	30 units per cc.
4½ units per cc. blood serum	6 hours	36 units per cc.

A clinical application was then made on twenty-one cases of diphtheria, twelve of which received antitoxin intraperitoneally and nine intramuscularly.

The twelve cases receiving intraperitoneal injections may be briefly outlined as follows:

Case 1. R. C. two years. Weight, thirty-five pounds, four days ill, membrane covering both tonsils. Bilateral anterior cervical adenitis. 10,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin 4 hours—0.75 units
Recovered. 6 hours—2.0 units

Case 2. I. G. fourteen years. Weight, seventy pounds, four days ill, membrane covering tonsils. Slight bilateral anterior cervical adenitis. 14,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin 1 hour —0.25 units
3 hours—0.33 units
12 hours—1.75 units
Recovered. 24 hours—5.00 units

Case 3. S. J. fifteen months. Weight, twenty-six pounds, five days ill, pharynx negative to inspection. Temperature 104°. Pronounced retractions—complete impairment of phonation—color poor—physical signs of bronchopneumonia—almost moribund. Laryngoscopic examination—marked supraglottic edema—membrane on ventricular bands. 10,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin 3 hours—0.5 units

Intubated one hour after admission. Died thirty-five hours later.

Autopsy (portion of report)—Small needle mark about one inch below umbilicus—no evidence of this on peritoneal surface—no congestion of surrounding tissues. Peritoneum smooth and glistening, free from adhesions. No injection of vessels. Peritoneal cavity contains no free fluid and intestines appear normal. Lungs—consolidation of both lower lobes, left upper and right middle lobes, with many firm nodules—purulent exudate from bronchioles.

Diagnosis—Laryngeal diphtheria and extensive bronchopneumonia.

Case 4. G. M. four years. Weight, thirty-seven pounds. ? days ill—membrane over posterior half of tonsils and posterior pillars. 10,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin 2 hours—0.33 units
4 hours—0.75 units
Urticarial rash on seventh day. 12 hours—2.00 units
No rise in temperature. No constitutional symptoms.
Recovered.

Case 5. S. S. two and a half years. Weight, thirty-eight pounds, five days ill—extensive sloughing membrane over tonsils and uvula—profuse purulent nasal discharge—bilateral anterior cervical adenitis. 10,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin Before—None
2 hours—0.33 units
Recovered. 14 hours—2.50 units

Case 6. H. N. five years. Weight, thirty-nine pounds, five days ill, toxic extensive membrane over both tonsils, pillars, and uvula. Bilateral units with 8-10 cc. saline by gravity.

Blood serum antitoxin Before—None
2 hours—0.5 units
4 hours—0.75 units
Recovered. 18 hours—4.00 units

Case 7. W. W. three and a half years. Weight, thirty-five pounds, three days ill, serous nasal discharge, membrane covering both tonsils, bilateral anterior cervical adenitis. 10,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin Before—0.04 units
5 hours—1.50 units
Recovered. 16 hours—4.00 units

Case 8. R. O. six years. Weight, forty pounds, three days ill, membrane covering both tonsils, bilateral anterior cervical adenitis, soft systolic mitral murmur. 10,000 units with 8-10 c.c. saline by gravity.

Blood serum antitoxin Before—0.04 units
7 hours—1.50 units
Recovered. 16 hours—4.00 units

Case 9. V. C. five months. Weight, fourteen pounds, ? days ill, profuse serosanguineous nasal discharge. 6500 units with 8-10 cc. saline by gravity.

Blood serum antitoxin Before—0.07 units
Recovered. 6 hours—6.5 units

Case 10. W. G. six years. Weight, fifty-five pounds, five days ill, extremely toxic on admission, sloughing putrid exudate over tonsils and pillars, bilateral anterior cervical adenitis, veins small and partially collapsed. 14,000 intraperitoneal with few cc. saline by gravity. Pulse rather weak and thirty-six hours after admission dropped suddenly from 130 to 80. Death twelve hours later.

Blood serum antitoxin Before—None
24 hours—7 units
48 hours—6 units

Autopsy (portion of report)—Peritoneum smooth and free from adhesions—no signs of inflammation.

Summary—Acute hemorrhagic myocarditis, pulmonary edema and congestion. Acute parenchymatous degeneration of liver and kidneys. Congestion of viscera.

Diagnosis—Septic diphtheria.

Case 11. J. D. eight years (?). Weight, forty-six pounds. Positive cultures but no clinical evidence of diphtheria. 10,000 units with 8-10 cc. saline by gravity.

Blood serum antitoxin Before—0.06 units
24 hours—3.00 units
36 hours—6.00 units
Recovered. 72 hours—3.00 units

Case 12. C. H. eight years. Membrane on right tonsil. 10,000 units with 8-10 c.c. saline. No determination.

Of this group of twelve, two were nearly moribund on admission and died with thirty-six and forty-eight hours respectively. Antitoxin was given intraperitoneally to these only because of the inability to give it by vein. They are reported with autopsy findings which show death to be due in one case to laryngeal diphtheria and extensive bronchopneumonia; in the other, to septic diphtheria with hemorrhagic myocarditis. In both, the peritoneum was free from any pathologic condition.

Ten cases recovered without complications and it is interesting to note that in most of these the membrane disappeared at about the same time that it did in a number of intravenous cases observed simultaneously.

The intramuscular group includes cases of nasal diphtheria, very mild tonsillar diphtheria and carriers.

They were selected to conform as nearly as possible in age and weight to the intraperitoneal cases. A brief statement of each is given:

Case 13. G. W. five years. Weight, thirty-six pounds. Positive culture, no clinical evidence of diphtheria, 10,000 units intramuscularly.

	Blood serum antitoxin	Before—0.09 units
		5 hours—0.14 units
Recovered.		7 hours—0.33 units

Case 14. T. O. four years. Weight, thirty-seven pounds. Very small amount of membranous exudate on both tonsils. No adenitis. 10,000 units intramuscularly.

	Blood serum antitoxin	Before—0.04 units
		2 hours—0.10 units
Recovered.		4 hours—0.16 units

Case 15. R. Y. fifteen years. Weight, ninety pounds. Positive culture but no clinical evidence of diphtheria. 10,000 units intramuscularly.

	Blood serum antitoxin	Before—0.07 units
		3 hours—0.07 units
Recovered.		6 hours—0.14 units

Case 16. R. K. three years. Weight, thirty pounds. Positive culture, serosanguineous nasal discharge, no membrane. 10,000 units intramuscularly.

	Blood serum antitoxin	Before—None
Recovered.		6 hours—0.14 units

Case 17. G. C. eleven years. Weight, fifty pounds. Positive cultures. No clinical evidence of diphtheria. 10,000 units intramuscularly.

	Blood serum antitoxin	Before—0.12 units
		6 hours—0.20 units
		12 hours—1.50 units
		24 hours—2.50 units
Recovered.		48 hours—5.00 units

Case 18. F. T. five years. Weight, thirty-seven pounds. Serosangineous nasal discharge, positive culture. 10,000 intramuscularly.

	Blood serum antitoxin	Before—0.07 units
		7 hours—0.20 units
Recovered.		24 hours—3.00 units

Case 19. R. G. seven years. Weight, forty-two pounds. No clinical evidence of diphtheria. 10,000 units intramuscularly.

Blood serum antitoxin. Before—0.125 units
12 hours—0.90 units
24 hours—2.00 units
48 hours—3.00 units
Recovered. 72 hours—4.00 units

Case 20. F. K. seven years. Weight, forty-five pounds. No clinical evidence of diphtheria. 10,000 units intramuscularly.

Blood serum antitoxin 24 hours—1.0 units
48 hours—1.5 units
60 hours—2.0 units
Recovered. 72 hours—2.5 units

Case 21. D. McN. No clinical evidence of diphtheria. 10,000 units intramuscularly.

Blood serum antitoxin Before—None
24 hours—1.0 units
36 hours—2.5 units
48 hours—3.5 units
Recovered. 96 hours—3.0 units

On fifty bleedings, taken at various time-intervals from the patients receiving intraperitoneal and intramuscular injections, a total of one hundred and thirty-four tests were performed.

Table 6 gives figures representing in most cases an average of several bleedings.

TABLE 6.

COMPARATIVE ABSORPTION OF ANTITOXIN BY THE INTRAMUSCULAR AND INTRAPERITONEAL METHODS

Intramuscular	Hours	Intraperitoneal
....	1	............0.38 units per cc. blood serum
0.10............	2	............0.42 units per cc. blood serum
0.18....	3	............0.43 units per cc. blood serum
0.17............	4	............0.79 units per cc. blood serum
0.15............	5	............1.50 units per cc. blood serum
0.21............	6	............2.30 units per cc. blood serum
0.34............	7	............1.70 units per cc. blood serum
1.66............	12	............2.37 units per cc. blood serum
....	14	............2.71 units per cc. blood serum
....	16	............4.00 units per cc. blood serum
....	18	............4.57 units per cc. blood serum
2.30............	24	............6.5 units per cc. blood serum
		* * *
2.70............	36	............7.88 units per cc. blood serum
		* * *
4.20...........	48	............6.75 units per cc. blood serum
		* * *
2,8*............	60	....
		* * *
4.00............	72	............3.94 units per cc. blood serum
		* * *
3.2	96	....

(The dose was 10,000 units except in two cases. Proper corrections have been made for these as well as for standard weight of thirty-five pounds.)

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*One bleeding only—24 hours—1 unit; 48 hours—1½ units; 60 hours—2 units; 72 hours—2½ units.

Several interesting points can be deduced from the preceding figures. To begin with, the most striking difference in the two methods is manifest in the first seven hours, the time of greatest importance. Within an hour after intraperitoneal injection, the blood is appreciably antitoxic, and up to seven hours the absorption is about five times that of intramuscular.

The peak of absorption for the intraperitoneal method lies apparently in the vicinity of thirty-six hours, whereas for the intramuscular it is somewhere between forty-eight and seventy-two hours. (In cases with successive bleedings it was nearer seventy-two.) From these points the values decrease as the serum is eliminated.

The high figure on the intraperitoneal side at six hours as compared to seven hours is noteworthy. It is explained by Case 9 which was included in the average for six hours. This case was one of nasal diphtheria in an infant of five months, which weighed only fourteen pounds. Although it received only 6,500 units its blood serum at six hours showed an actual antitoxin content of 6.5 units per cc. This figure when corrected for standard weight (thirty-five pounds) to 3.23 is still much higher than those observed in older children and suggests, as might be expected, that the younger the child, the more rapidly will antitoxin be taken up from the peritoneum. This is significant because it is among the younger children that the procedure seems particularly applicable.

To illustrate these points as well as to facilitate comparison of all four methods a graphic chart is appended.

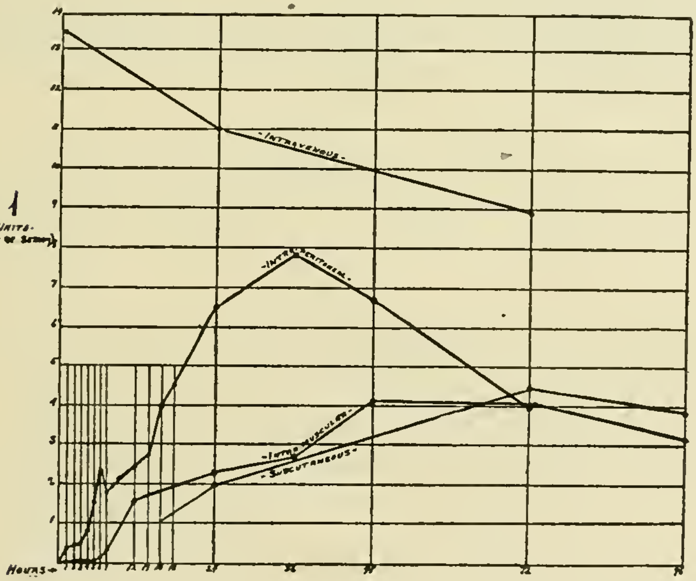

Fig. 1. Graphic representation of absorption—units per cc. blood-serum.

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SUMMARY AND CONCLUSIONS.

1. A new method of administering antitoxin, namely, the intraperitoneal, is suggested for selected cases of diphtheria.

2. For infants and young children, the simplicity of technique in methods other than the intravenous is emphasized.

3. Local discomfort, an annoying factor in the subcutaneous and intramuscular methods, is absent in the intraperitoneal and intravenous methods.

4. General reactions are noted as a possible hazard, greater in the intravenous than in the other methods.

5. Absorption by the intraperitoneal route does not, of course, approach the intravenous where antitoxin is delivered directly into the blood stream. Early absorption of antitoxin, however, as shown by tests on the blood after intraperitoneal injection, is approximately five times greater than after intramuscular injection. Within an hour it is more than sufficient to neutralize the circulating toxin in the blood of any case of diphtheria.

6. Absorption by the intraperitoneal method is more rapid in infants than it is in older children. It is among the former that the procedure finds its greatest use.

7. Intraperitoneal administration of antitoxin is a safe and simple procedure. It is particularly applicable: (1) In advanced cases where suitable veins for intravenous administration are lacking. (2) In moderately advanced cases in which intravenous injection is not considered necessary or advisable but where more rapid absorption is desired than can be obtained by the intramuscular method.

I wish to express my indebtedness to Dr. Park and Dr. Banzhaf for their kind suggestions and advice in this work and to Mr. Greenwald for his technical assistance.

For bibliography see the original article.

THE EFFECT OF PEPTONE ON THE TOXIGENIC PROPERTY OF B. DIPHTHERIAE NO. 8

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Harriet Leslie Wilcox

It is known to all laboratory workers who are familiar with the production of diphtheria toxin that the strain of B. diphtheriae used in obtaining a potent toxin is called No. 8. This strain was isolated by Dr. Williams in the summer of 1895 and so stable has been its toxigenic property that it is used, not only in the serum laboratories of the United States but in those of England and Europe as well.

The idea that different cultures of this strain should vary in their toxic productions was not entertained up to about 1916. However, from time to time, requests for new transplants of No. 8 had been received at this laboratory from other workers who said that their cultures had lost the ability to produce a potent toxin. Previous to 1914, our strain was still producing a potent toxin, though with some irregularities in strength.

More recently and coincident with the reports from other laboratories. the potency of the toxin produced at the Bureau of Laboratories has shown a marked decline. The average toxicity of the years 1914, 1915 and 1916 was 1:200 (or less) as compared with an average potency of 1:350-1:400 for the years 1911, 1912 and 1913.

In the summer of 1916 I was privileged to work a few weeks at the Pasteur Institute in Paris and to observe the preparation and inoculation of the diphtheria toxin broth. This procedure consisted of seeding Martin peptone broth[1] in Fernbach flasks with fairly large amounts of broth culture of No. 8. This culture had been taken to the Pasteur Institute in 1896 by Dr. Williams. The usual routine for the preparation of diphtheria strain was as follows:

> The stock culture was kept on coagulated ox-serum while the other culture was cultivated entirely on Martin peptone broth and was transferred three times weekly. The culture on serum was subcultured every four weeks and was not used for toxin production unless the broth culture became contaminated; in such case a fresh culture in Martin peptone broth was made from the serum culture. How often recourse to this culture was made, I do not know, but from the careful technic employed one would say, rarely, if ever.

On my return several experimental preparations of diphtheria toxin were made. The method of the Pasteur Institute was used as well as their culture of No. 8 which had been brought back to New York. The results were so superior to those obtained by employing Witte peptone broth and our own culture of No. 8 that in January, 1917, Martin peptone broth inoculated with the Pasteur culture of B. diphtheriae No. 8 was adopted as our method of toxin production. Since we were following as closely as possible the Pasteur method, we used the same procedure for the propagation of the culture of B. diphtheriae.

Comparative tests of our culture of No. 8 with that of the Pasteur No. 8 were made at Dr. Williams' request. The first two or three tests showed such marked difference in favor of Pasteur No. 8 that we decided to discover whether, after a cultivation of several months or longer on Martin peptone broth, our No. 8 culture would produce as high a toxin in Martin peptone broth as the Pasteur No. 8.

To differentiate between these two cultures of the same strain of No. 8 we have called the culture which has been propagated exclusively at the Bureau of Laboratories, Research No. 8, whereas the culture obtained from Pasteur Institute is designated as Pasteur No. 8. These two cultures were inoculated in Martin peptone broth Dec. 28, 1916, and transferred three times weekly in this medium.

At about the same time these cultures were inoculated similarly into Witte peptone broth, Research No. 8 being planted Dec. 23, 1916, and Pasteur No. 8 Dec. 28, 1916, and were transferred three times weekly.

In the summer of 1919 a second culture of No. 8 was obtained from the Pasteur Institute and this was planted Aug. 19, 1919, in Martin peptone broth, Berna peptone broth and Witte peptone broth; it was designated as Pasteur 2 No. 8.

The records of these cultures, that is, the date each was planted in a different medium, the number of transfers weekly, together with the date and result of the planting to determine purity, were kept on separate cards.

In order to obviate any possibility of the substitution of one medium for another while making the subcultures, the following procedure was adopted:

The cultures Research No. 8, Pasteur No. 8, and Pasteur 2 No. 8 in the Martin peptone broth were kept in a different container than the same cultures in the other peptone broths. At the time of transferring only one container with its respective broth tubes was on the work table, thus avoiding any possibility of a Witte peptone broth tube being substituted for a Martin peptone broth tube and vice versa.

The dilutions of the filtered cultures for the animal inoculation were made in 0.8% NaCl. In the tables in which the toxicity is given as being below 1:100 or 1:200, etc., it indicates that those were the dilutions inoculated and that they did not kill the test animals, or if they did so, not within the time limits. When a toxin is stated to be of a given strength it means that the given dilution killed a 250 gm. guinea-pig within the four days with the classical toxic symptoms. Occasionally it was not possible to obtain pigs weighing 250 gm., but in those tests made with heavier pigs, the surplus weight was always discounted in estimating the toxicity. No guinea-pigs weighing below 230 gm. were inoculated.

Martin Peptone Broth

The comparative tests of Research No. 8 and Pasteur No. 8 in Martin peptone broth are given in table 1. It is to be recalled that the cultures were subjected to cultivation in this broth, were transferred three times weekly and were then cultivated under identical conditions. To give the reader the

TABLE 1.

TOXICITIES PRODUCED IN MARTIN PEPTONE BROTH BY THREE DIFFERENT CULTURES OF B. DIPHTHERIAE NO. 8 AFTER CULTIVATION IN THE SAME BROTH; TRANSFERS MADE THREE TIMES A WEEK

Date of Test	Research No. 8 Cultivation Begun Dec. 28, 1916	Pasteur No. 8 Cultivation Begun Dec. 28, 1916	Pasteur 2 No. 8 Cultivation Begun Aug. 19, 1919
Jan. 19, 1917	1:100	1:400	Not tested
May 31, 1917	Below 1:200	1:400	Not tested
June 30, 1917	1:300	1:500	Not tested
Oct. 11, 1917	Below 1:100	1:300	Not tested
Oct. 19, 1917	1:150	1:800	Not tested
Feb. 2, 1918	1:100	1:200	Not tested
Sept. 19, 1919	1:200	1:600	1:600
Oct. 2, 1919	Below 1:100	Below 1:200	Below 1:200

approximate number of generations before the different tests were made, the date of starting the cultures in the different broths is given in the tables.

It will be seen that in one test only Research No. 8 approximated Pasteur No. 8 in its toxic production. In the experiment dated May 31, 1917, Research No. 8 gave a toxicity of 1:300 as compared with a toxin of 1:400 produced by Pasteur No. 8. The greatest difference in their toxigenic powers was shown in the test dated Oct. 19, 1917, when the M L D of Research No. 8 was 1:150 in contrast to a potency of 1:800 produced by Pasteur No. 8. Subsequent comparative tests showed a diminution in the toxigenic production by both cultures in Martin peptone broth.

The first test comparing the three cultures, Research No. 8, Pasteur No. 8 and Pasteur 2 No. 8, was made Sept. 19, 1919. The results showed that the two Pasteur cultures gave a toxicity of 1:600 whereas the Research No. 8 gave a low M L D of 1:200. The last comparative test in Martin peptone broth was made in October, 1919, but the toxicities in this test were so low that only negative results were obtained.

WITTE PEPTONE BROTH

The number of tests made using Witte peptone broth was limited, due to the scarcity of the peptone. The broth used in these tests was made according to the formula recommended by Theobald Smith for obtaining a potent toxin. As in the test made with Martin peptone, the three cultures were transferred in the Witte broth 3 times weekly. The period of cultivation may be judged from the dates in table 2 in which the results are given.

TABLE 2.

TOXICITIES PRODUCED IN WITTE PEPTONE BROTH BY THREE DIFFERENT CULTURES OF B. DIPHTHERIAE No. 8 AFTER CULTIVATION IN THE SAME BROTH; TRANSFERS THREE TIMES A WEEK

Date of Test	Research No. 8* Cultivation Begun Dec. 23, 1916	Pasteur No. 8 Cultivation Begun Dec. 28, 1916	Pasteur 2 No. 8 Cultivation Begun Aug. 19, 1919
Jan. 19, 1917	Below 1:100	1:400	Not tested
Oct. 26, 1917	1:150	1:700	Not tested
Sept. 15, 1919	1:100	1:400	1:250
Mar. 12, 1920	Below 1:100	Below 1:100	Not tested
May 27, 1921	Below 1:100	Below 1:200	1:450

* This culture had been carried on in Witte broth since 1895 and transferred every other day as the routine procedure.

It will be noticed that despite the fact that Research No. 8 had been cultivated in a medium containing Witte peptone practically ever since its isolation in 1895, its toxigenic property was much less in the first three tests than that of Pasteur No. 8. The test made May 27, 1921, was a great surprise for it was the first time that Pasteur 2 No. 8 had given a higher potency than Pasteur No. 8. Whether this difference would have been demonstrable in the March 12, 1920, test it is impossible to say, but it is evident that the toxigenic property of Pasteur No. 8 in Witte broth had deteriorated. Further reference to this will be made in the following pages.

BERNA PEPTONE BROTH

It was stated in the foregoing that Martin peptone broth was used during the year 1917 at the Bureau of Laboratories for the routine production of diphtheria toxin, with satisfactory results. In the early months of 1918, however, the potency of the toxin was irregular, and in the late spring several successive preparations of the broth gave too low a toxin for practical use. At this time we had just begun to use the Berna peptone for the production of tetanus toxin, and the idea occurred to us that this peptone might be satisfactory for obtaining a potent diphtheria toxin. Therefore a small amount of broth was made according to the method of Theobald Smith, but with the Berna peptone instead of Witte. The result of this test was very gratifying as a M L D of 1:350 was obtained. No comparative tests of Research No. 8 and Pasteur No. 8 were made at this time as I began a year's leave of absence. During this time all of the cultures in the different mediums were carried out on by Miss Alice G. Mann whose cooperation is greatly appreciated. The two cultures, Pasteur No. 8 and Research No. 8 were planted in Berna peptone broth and were transferred three times weekly.

On my return from France in 1919 the comparative tests in Berna peptone broth of the two cultures, Research No. 8 and Pasteur No. 8 were made, together with the second culture of No. 8 that had been obtained from the Pasteur Institute in the summer of 1919 and which is spoken of in the test as Pasteur 2 No. 8. The results are given in table 3.

TABLE 3.

TOXICITIES PRODUCED IN BERNA PEPTONE BROTH BY THREE DIFFERENT CULTURES OF B. DIPHTHERIAE NO. 8 AFTER CULTIVATION IN THE SAME BROTH; TRANSFERS THREE TIMES A WEEK

Date of Test	Research No. 8 Cultivation Begun May 4, 1918	Pasteur No. 8 Cultivation Begun May 4, 1918	Pasteur 2 No. 8 Cultivation Begun Aug. 19, 1919
Aug. 22, 1919	Below 1:100	1:100	1:100
Sept. 13, 1919	Below 1:100	1:250	1:250
Sept. 15, 1919	1:100	1:500	1:500
Sept. 19, 1919	Below 1:100	1:500	1:400
Sept. 26, 1919	Below 1:100	1:100	1:100
Oct. 4, 1919	Below 1:100	1:100	1:100
Oct. 10, 1919	Below 1:100	1:250	1:250
Oct. 18, 1919	Below 1:100	1:150	Below 1:100

Four of the 8 tests given in table 3 showed decidedly the greater toxigenic property of the two Pasteur cultures over that of Research No. 8. The toxicities of the other four tests were so uniformly low that less definite comparisons were possible.

The toxin made from the Berna peptone broth was so inferior from October, 1919, through to the new year that efforts were made to discover whether there was a domestic peptone on the market which would be more satisfactory for diphtheria toxin production. A comparative test of Parke-Davis peptone and Berna peptone broths was made and inoculated with only Pasteur No. 8. The results were encouraging for the toxin made from

Parke-Davis broth had a M L D of 1:700 in contrast to a M L D of 1:200 obtained with Berna peptone broth. Accordingly, the three cultures were cultivated in broth made with Parke-Davis peptone. However, no comparative tests of the toxigenic powers were made until March, 1920. These and subsequent results are given in table 4 in which is also included three tests of the culture "American."

TABLE 4.

TOXICITIES PRODUCED IN PARKE-DAVIS PEPTONE BROTH BY THREE DIFFERENT CULTURES B. DIPHTHERIAE NO. 8 AFTER CULTIVATION IN THE SAME BROTH; TRANSFERS THREE TIMES A WEEK

Date of Test	Research No. 8 Cultivation Begun Jan. 1, 1920	Pasteur No. 8 Cultivation Begun Jan. 1, 1920	Pasteur 2 No. 8 Cultivation Begun Jan. 1, 1920	"American"*
Mar. 3, 1920.....	1:100	1:1000	Not tested	Not tested
Mar. 12, 1920.....	1:100	1:800	Not tested	Not tested
Nov. 16, 1920.....	1:300	1:900	Not tested	Not tested
Nov. 26, 1920.....	1:200	1:1000	1:700	1:1000†
Jan. 21, 1921.....	1:200	1:1000	1:300	1:700‡
April 7, 1921.....	1:100	1:1500	1:700	1:800

* This culture was obtained from Dr. Durand, Lyons, France, and was the B. diphtheriae No. 8 which had been sent him probably from the Pasteur Institute, Paris.

† This culture had been inoculated into Parke-Davis peptone broth for seven generations before seeding of toxin flasks.

‡ This had been in Parke, Davis broth for five generations before seeding of toxin flasks.

The marked differences in the toxigenic power of these strains were again in evidence. As far as was known, there was no explanation for the low toxicity of Pasteur's 2 No. 8 in the test made Jan. 21, 1921. This was 1:300 as against the M L D of 1:1,000 produced by Pasteur No. 8. In all the other comparative tests with the different peptones, the toxin made from Pasteur 2 No. 8 had corresponded very closely with the toxins made from Pasteur No. 8 with the exception of the experiment of Sept. 15, 1919, made in Witte peptone broth (table 2). Here Pasteur No. 8 produced a M L D of 1:400 as compared with a toxicity of 1:250 made from Pasteur 2 No. 8.

It is interesting to note that the "American" culture (table 4, No. 26, 1920) gave after only seven generations in Parke-Davis peptone broth a toxin with as high a potency as the Pasteur No. 8 which had been cultivated eleven months in that medium.

When it was evident that Research No. 8 produced consistently a very much lower toxin than either of the other strains, regardless of the length of cultivation in any particular broth, the thought occurred to us that it would be interesting to see what effect the different peptones may have had on the toxigenic power of Pasteur No. 8 after its cultivation in the different peptone broths. Accordingly, a preparation of Berna peptone broth was inoculated on July 23, 1920, with the Pasteur No. 8 culture which had been cultivated exclusively in Martin, Witte, Parke-Davis and Berna peptone broths. The day before the test was started, seed cultures in Berna peptone broth were planted from these cultures, as fairly large amounts of the seed cultures were used to inoculate the toxin flasks and we wished to obviate carrying over any of the other peptone broths into the Berna broth. The results of this test, together with the dates of first cultivation in the different broths, are given in table 5.

TABLE 5.

TOXICITIES PRODUCED IN BERNA PEPTONE BROTH BY PASTEUR NO. 8 AFTER CULTIVATION IN BROTHS MADE WITH DIFFERENT PEPTONES

Transplanted Three Times Weekly		Date of Test	Toxicity
First Transplant of Series	Cultivated in Broth with		
May 4, 1918	Berna peptone broth	July 23, 1920	1:500
Dec. 28, 1916	Witte peptone broth	July 23, 1920	Below 1:100
Dec. 28, 1916	Martin peptone broth	July 23, 1920	1:450
Jan. 1, 1920	Parke-Davis peptone broth	July 23, 1920	1:450

TABLE 6.

TOXICITIES PRODUCED IN WITTE PEPTONE BROTH BY PASTEUR NO. 8 AFTER CULTIVATION IN BROTHS MADE WITH DIFFERENT PEPTONES

Transplanted Three Times Weekly		Date of Test	Toxicity
First Transplant of Series	Cultivated in Broth with		
Dec. 28, 1916	Witte peptone broth	Dec. 9, 1920	1:150
Dec. 28, 1916	Martin peptone broth	Dec. 9, 1920	1:300
May 4, 1918	Berna peptone broth	Dec. 9, 1920	1:500

The results of the foregoing test show that the cultures of Pasteur No. 8, which had been cultivated exclusively in Berna, Martin and Parke-Davis peptone broths, gave satisfactory toxins. The culture, however, which had been transferred in Witte broth since Dec. 28, 1916, gave a potency of below 1:100.

Another test was made with these same cultures which had been cultivated in broths made with Witte, Berna and Martin peptone, but instead of the Berna peptone broth, Witte peptone broth was used. As in the former test, the seed cultures were planted in Witte peptone broth before the inoculation in the Witte toxin broth.

Here again the culture which had been in Witte broth gave the lowest toxicity. This test was repeated nine months later, on April 22, 1921 (table 7).

TABLE 7.

TOXICITIES PRODUCED IN WITTE PEPTONE BROTH BY PASTEUR NO. 8 AFTER CULTIVATION IN BROTHS MADE WITH DIFFERENT PEPTONES

Transplanted Three Times Weekly		Date of Test	Toxicity
First Transplant of Series	Cultivated in Broth with		
May 4, 1918	Berna peptone broth	April 22, 1921	1:800
Dec. 28, 1916	Witte peptone broth	April 22, 1921	Below 1:100
Dec. 28, 1916	Martin peptone broth	April 22, 1921	1:700
Jan. 1, 1920	Parke-Davis peptone broth	April 22, 1921	1:700

It will be seen that these results confirm the former test in the Witte peptone broth and also that in the Berna broth, in that the culture of Pasteur No. 8 which had been cultivated in the Witte broth gave the lowest toxicity.

Attempts will be made to confirm the results reported in this paper: (1) By subcultivating in Witte broth the culture Pasture No. 8 which has been propagated continuously in Parke-Davis peptone broth since January, 1920, and which has given regularly a potent toxin in this medium; and by comparing these two cultures from time to time in order to determine whether the toxigenic powers of the culture in Witte broth has been adversely affected while the culture kept in Parke-Davis peptone broth retains its ability to produce strong toxin.

(2) By subcultivating Pasteur No. 8, which has apparently lost its toxigenic power by cultivation in Witte broth, in Parke-Davis peptone broth to determine whether this culture after varying lengths of time in Parke-Davis broth may not regain its power to produce a potent toxin.

(3) By further cultivation of Pasteur 2 No. 8 in Witte broth to determine whether this culture will lose its toxigenic power in Witte peptone broth as Research No. 8 and Pasteur No. 8 have apparently done.

SUMMARY

The culture of research No. 8 which has been propagated exclusively in Witte broth since 1895 at the Bureau of Laboratories has apparently lost its power to produce a potent toxin in any of the broths tested.

The Pasteur cultures of No. 8 which were planted in Witte broth Dec. 28, 1916, has also apparently lost its ability to produce a potent toxin in both Witte and Berna peptone broths. The same culture kept in Berna, Martin and Parke-Davis peptone broth produces strong toxins in both the Berna and Witte broths.

The cultures of Research No. 8 which have been carried on in Berna, Martin and Parke-Davis peptone broths have not as yet recovered their powers to produce a potent toxin in any of the broths tested.

The toxigenic property of Pasteur 2 No. 8 culture has not as yet been affected deleteriously by the two years' cultivation (1919-1921) in Witte peptone broth.

CONCLUSIONS

It would seem in view of the foregoing results:

That Witte peptone broth had an inhibitory or destructive influence on the toxigenic powers of both Research No. 8 and Pasteur No. 8 cultures.

That Pasteur 2 No. 8 may be similarly affected after a still longer cultivation in Witte broth.

That the different preparations of Witte peptone received in this country just prior to 1914 may be responsible for this deleterious effect on the cultures. The fact that culture Research No. 8 from the time of its isolation in 1895 to 1912-1913 had been cultivated in Witte peptone broth without any signs of the lowering of its toxigenic property, as shown by the production of a potent toxin, tends to confirm this conclusion.

That it is possible that the different preparations of any peptone may vary in their effects on the toxigenic property of B. diphtheriae No. 8.

That the continuous cultivation of a culture of B. diphtheriae No. 8 in the same broth as that used for the toxin production is apparently not necessary for obtaining a potent toxin, is shown by the "American" strain of No. 8, which after only seven generations in Parke-Davis broth gave as potent a toxin in that medium as Pasteur No. 8 which had been cultivated for eleven months in Parke-Davis broth.

For bibliography see the original article.

THE EFFECT OF FREEZING AS REGARDS TOXICITY ON DIPHTHERIA TOXIN-ANTITOXIN MIXTURE

Edwin J. Banzhaf and Chas. K. Greenwald

On January 2. 1922, a number of 50 c.c. bottles of toxin-antitoxin mixture No. 38 were sent to a Brooklyn Public School where Dr. Schroeder was immunizing the children. After the day's work was finished the excess toxin-antitoxin mixture (about four 50 cc. bottles) was left in the school room. The following day she took more of the same preparation to the school and, on reclaiming the four left overs. found them to be frozen solid and had them returned to the laboratory on the morning of January 4. When they arrived they were still frozen solid. We kept them at room temperature until they were thawed. After thawing and shaking they were very turbid.

Tests were put on guinea pigs the same day as follows, and with the following results:

Dose injected	*Reactions*	*Results*
1 cc.	No paralysis	Discharged
1 cc.	No paralysis	Discharged
3 cc.	No paralysis	Discharged
3 cc.	No paralysis	Discharged
5 cc.	Slight paralysis	Dead 23 days
5 cc.	Slight paralysis	Dead 27 days Pneumonia

Preparation No. 38 was a five L+ mixture and was ready for Berkefelding April 18, 1921. Tests were put on the following day as follows: The two cc. pigs died from complete paralysis after 24 and 28 days. The five cc. pigs died within 11 and 13 days.

The last test was made on Dec. 15, 1921 on the bottled lot that was used by Dr. Schroeder and was as follows:

Dose injected	*Reactions*	*Results*
2 cc.	Marked paralysis	Recovered
2 cc.	Marked paralysis	Recovered
5 cc.	Complete paralysis	Dead 21 days
5 cc.	Complete paralysis	Dead 21 days

No further tests were made as these convinced us at that time that nothing serious resulted from a solid freezing other than a diminution of the toxicity and also of the immunity value.

On January 8th, 1924, a physician from Atlanta, Georgia, wrote that he had four vials of preparation No. 91 toxin-antitoxin 0.1 L+ that had been frozen and asked for others. We wrote him to return those that were frozen. When the vials were received only a very slight haze could be detected when compared with other vials of the same preparation. Guinea pigs injected with five cc. showed no symptoms of paralysis and on schicking seven weeks later showed no immunity. This preparation No. 91, on Nov. 26, 1923, tested as follows: One cc. pigs, complete paralysis with death in 19 and 21 days. Three cc., death in 9 and 17 days. Five cc., death in four and six days.

On or about the third of February we heard from Massachusetts that a lot of toxin-antitoxin mixture had become toxic after freezing.

We immediately froze a number of our preparations selecting those that were prepared with different lots of toxin. The toxin-antitoxin mixtures which we froze were 3, 1, 0.5 and 0.1 L+.

The procedure in all the freezing experiments in the laboratory were as follows:

One hundred cc. samples were taken and placed 50 cc. in each of two four-ounce bottles which were well stoppered and sealed. As one of each pair was to be frozen they were marked for identification with a diamond pencil. The samples with the remaining amounts were placed in the refrigerator for controls. At about 3 P. M. a 36 quart container was half filled with cracked ice and salt, the bottles placed therein and packed separately with ice and salt and the container then filled with ice and salt. The container cover was adjusted and the whole placed outside on the fire escape. At ten in the morning there was always ice left in the container. The contents of the bottles were always found to be frozen in a solid mass. These were called the 18 hour freezings. Thawing was allowed to take place at room temperature. All the products, after thawing, with the exception of autoclaved media which was ready for planting, showed precipitates. Those of the toxin-antitoxin mixtures were slight for the 0.1 L + and increased with the more concentrated mixtures. On shaking the 0.1 L + showed a very slight haze; the 1 L + a definite turbidity and the 3 L + was very turbid. Tests were made the same day except in cases stated.

The 3 L + mixture that was frozen was prepared in June 1922. At that time one cc. doses caused complete paralysis in guinea pigs and death in from 15 to 23 days. One year later 3 cc. caused paralysis with death in 30 days and 5 cc. doses paralysis with death in 20 to 33 days. After freezing, 5 cc. doses in guinea pigs failed to cause the slightest sign of paralysis. Twenty-five cc. of the frozen mixture was centrifuged, twenty cc. pipetted off and the precipitate rubbed up in the remaining five cc. This was injected into a guinea pig. The pig showed no sign of illness or paralysis. These pigs were schicked seven weeks later and showed no immunity. As this mixture had not been tested for more than seven months, five cc. doses of the sample before freezing were injected. These pigs showed a definite paralysis. A one L + mixture was prepared in November 1922 and was very toxic. One cc. pigs died acutely within three days. After freezing, five cc. injected into guinea pigs failed to show paralysis. Seven weeks later the same frozen sample was retested. Five cc. doses showed no reaction.

Sample before freezing, tested on March 19, 1924, gave the following: One cc. doses, death in 4½ days and 5½ days. Five cc. pigs died acutely in two and a half days. The freezing of this acutely toxic mixture of 1 L + toxin-antitoxin caused a complete loss of toxicity.

A one-half L + mixture was prepared in November, 1922 and was also acutely toxic in guinea pigs with one cc. doses. Tests again December 26, 1923, one cc. pigs died in 10 and 12 days. Three cc. pigs died in 5 days and five cc. pigs in 3 days.

After freezing, 5 cc. doses failed to produce symptoms of paralysis. Schicked after seven weeks the reading showed only a slight immunity. Here again the freezing of a toxic mixture caused practically a complete loss of toxicity.

The tests recorded thus far on freezing were made on mixtures that were more than a year old and in two they were still acutely toxic with 5 cc. doses at the time of freezing.

On February 7, 1924, Dr. Cornman, Director of the Gilliland Laboratory, froze their 0.1 L + toxin-antitoxin mixture No. 40 for 24 hours. After thawing he injected six pigs with one cc. each and six pigs with 5 cc. each. His one cc. pigs showed no evidence of paralysis. All of his five cc. showed some paralysis followed by recovery. At our request he sent the twelve pigs to us. We shicked the 6 one cc. pigs seven weeks after their injection. They showed no immunity. This mixture was prepared in November, 1923, and his last retest was on December 18, 1923, as follows:

Two one cc. pigs showed definite paralysis followed by recovery, two others complete paralysis with death in 26 and 28 days. Three cc. pigs showed complete paralysis and death in 16 and 18 days. Five cc. pigs died in 9 and 12 days.

On February 9 we received from Dr. Benjamin White twenty-four vials of the same 1 L + mixture of toxin-antitoxin which, when frozen in Massachusetts, had become toxic. These vials were labeled No. 101c and each contained 20 c.c. We numbered them from 1 to 24 consecutively. The first two were pooled and likewise the next two. Samples were taken from each pooling and tested. The remainder of each pooling was frozen together with sample of our 1 L + and 0.5 L+ previously reported; a 0.1 L + No. 96, the concentrated toxin used for preparing the mixture and the concentrated antitoxin used in the mixture, all of these were frozen in the same container and in the same manner previously described. The one 1 L + and 0.5 L + again lost their toxicity. The five cc. pigs failed to show any paralysis. Our 0.1 L + No. 96 with two cc. doses showed no evidence of paralysis. One five cc. died in 13 days with lungs consolidated, the other had some questionable symptoms of paralysis and died after 31 days with lungs consolidated. This mixture, No. 96, was prepared November 23, 1923 and tested on the same day as follows: One cc. pigs died in 10 to 14 days; three cc. pigs in four and a half days to five days; five cc. in two and a half days. Tested on February 13, 1924, the sample before freezing killed five cc. pigs in five and a half to eight and a half days. Retested on March 14, one cc. pigs died from complete paralysis in 26 and 27 days. The five cc. pigs died in seven and eleven days. As can be seen the toxicity of this mixture was greatly reduced after freezing.

The concentrated diphtheria antitoxin tested before freezing 1050 units per cc. This is the antitoxin that is being used in preparing our mixtures. After freezing the value remained the same. The aged concentrated toxin sample before freezing had an M.L.D. value of 0.012 and after freezing was 0.07, showing a loss of about 80 per cent. toxin. That diphtheria toxin lost considerable of its value and that diphtheria antitoxic serum retained its value on freezing was known from the work of one of us seventeen years ago.

The Massachusetts 1 L + mixture marked No. 101c gave the following tests before freezing.

	Weight of Pigs in Grams	*Dose in cc.*	*Reaction*	*Results*	*Remarks*
Pooled No.	245	1	Complete paralysis	Died 26 days	
1 & 2 sample	255	1	Complete paralysis	Died 19 days	Lungs partly consolidated
before freezing	320	5		Died 4 days	Lungs consolidated
	290	5		Died 9 days	
Pooled No.	240	1	Complete paralysis	Died 24 days	
3 &4 sample	245	1	Complete paralysis	Died 27 days	
before	300	5		Died 11 days	
freezing	310	5		Died 15 days	

These tests proved that before freezing the mixture was just right for the best results for active immunity.

After the freezing the tests were as follows:

	Weight of Pigs in Grams	*Dose in cc.*	*Reaction*	*Results*	*Remarks*
	245	1		Dead 6½ days	
Pooled	235	1		Dead 6¾ days	
No. 1 & 2	245	1		Dead 8½ days	
after	320	5		Dead 2¾ days	
freezing	280	5		Dead 2 days	
	320	5		Dead 2½ days	
Pooled	235	1		Dead 6½ days	
No. 2 & 3 after	240	1		Dead 1½ days	Lungs consolidated
freezing	250	1		Dead 3 days	Partly consolidated lungs
	310	5		Dead 2½ days	
	320	5		Dead 2 days	
	320	5		Dead 1½ days	

These tests corroborate the findings of Dr. White. He reported that the Massachusetts 1 L + mixture became toxic after freezing.

In order to learn if extreme cold, that is, temperatures below zero Fahrenheit would cause our mixtures to become toxic and what effect it would have on other products, we arranged through the coöperation and courtesy of the Brooklyn Bridge Freezing and Cold Storage Company to have space for prolonged freezing at 16° below zero Fahrenheit.

For this low freezing, we prepared on February 8 a 1 L + mixture with 0.9 unit of antitoxin per L +. Only the five cc. doses were injected into pigs and these died acutely in three and a half and four and a half days. Desiring to have a freshly prepared 1 L + mixture more toxic than this, another was prepared on February 11 with 0.8 of a unit per L +. The one cc. dose pigs died in seven days and the five cc. pigs in two and three days. Samples of these two preparations together with the following were placed in four crates and sent to the cold storage on February 13: samples of an aged diphtheria toxin; a diphtheria toxoid (a toxin with 0.1 % forymaldehyde and incubated); a 0.1 L + mixture No. 92 prepared in November; the Massachusetts 1 L + mixture No. 101c, and samples of a 1 L + mixture No. 91 which Miss Kirkbride of the New York State Laboratory sent us.

One crate was removed after a 24 hour freezing, the recorded temperature was 16° below zero Fahrenheit; the second was removed after 48 hours; the third after five days and the fourth after eleven days. As our supply of guinea pigs was very low we could not test the first three crates as they were removed. The samples in the fourth crate were tested the second day after removal. The aged toxin sample lost more than 80 per cent. of its toxin. During December the toxoid (modified toxin) caused with one cc. doses complete paralysis and death in 25 days. After freezing one cc. doses caused symptoms of paralysis with recovery.

The 0.1 L + mixture No. 92 tested on December 10 as follows: one cc. pigs died in 15 to 18 days, three cc. pigs five to seven days, and the five cc. pigs in two and a half to five and a half days. After the eleven days freezing, five cc. pigs failed to show convincing symptoms of paralysis.

The freshly prepared 1 L + mixture with 0.9 unit per L +, after freezing, lost considerable toxicity. The five c.c. dose pigs died in 22 to 26 days. Both pigs showed lungs partly consolidated. Both showed, however, definite symptoms of paralysis. Samples of this mixture before freezing were tested again on March 26, 1924. The five cc. pigs died in 5 to 14 days The second freshly prepared 1 L + mixture with 0.8 unit per L + also lost considerable toxicity. The five cc. pigs developed complete paralysis and died after 25 and 27 days. Samples of this mixture before freezing were retested on March 26, 1924. The five cc. doses killed acutely in three and three and a half days.

The 1 L + New York State Laboratories mixture No. 91 tested on February 11, showed the following results: One one cc. pig died in 14 days, lungs consolidated. Two one cc. pigs died in 39 days. One five cc. pig died in 14 days, the other became completely paralyzed and died in 24 days. After the eleven-day freezing, the five cc. pigs showed some definite paralysis with recovery.

The Massachusetts 1 L + after the eleven-day freezing at 16° below zero Fahrenheit was even more toxic than with the 18-hour laboratory freezing. Only doses of 1 cc. were given, the results follow; at the same time these tests were made a duplicate number of pigs were injected in the leg

No. on Sample 101 *c.*	*Weight of Pigs in Grams*	*Dose in cc.*	*Results*
9	245	1	Dead 3½ days
9	250	1	Dead 2½ days
10	250	1	Dead 3½ days
10	250	1	Dead 2½ days
11	250	1	Dead 3 days
11	255	1	Dead 2½ days
12	250	1	Dead 2½ days
12	250	1	Dead 3½ days
9	245	1 c.c. + 100 units in leg, no reaction	
9	245	1 c.c. + 100 units in leg, no reaction	
10	240	1 c.c. + 100 units in leg, no reaction	
10	250	1 c.c. + 100 units in leg, no reaction	
11	250	1 c.c. + 100 units in leg, no reaction	
11	255	1 c.c. + 100 units in leg, no reaction	
12	235	1 c.c. + 100 units in leg, no reaction	
12	245	1 c.c. + 100 units in leg, no reaction	

muscle with 100 units of antitoxin and the one cc. test dose injected subcutaneously in the usual place.

Unfortunately immediate further tests could not be made as there was a shortage of guinea pigs. It was our desire to conserve those we had to determine if diluted antitoxin lost any of its value on freezing. We therefore diluted antitoxin with saline solution to three units per cc. to correspond to the number of units in the old 3 L + mixture and also made a dilution with saline solution to one unit per c.c. to correspond with the amount in the 1 L +. We also heated to 80° C for twenty minutes, the toxin used in our mixtures and diluted this with saline solution to conform to a 3 L + per cc. and added three units of antitoxin per cc. A dilution was also made to contain 1 L + and one unit was added per cc. to correspond to the amount of antitoxin in a 1 L + mixture.

After freezing and thawing, a small amount of a light fluffy precipitate was found in the saline dilution containing 3 units per cc. and a smaller amount of a like precipitate in the one unit per cc. Both solutions were shaken and placed in the refrigerator over night. The next day a small amount of the clear solution was removed and the remainder shaken. Both the clear and the turbid solutions of each dilution were tested. The clear solutions and the slightly turbid solutions showed the same antitoxic value as before freezing, proving that the small amount of precipitate was not antitoxin.

Samples of the diluted heated toxin solutions containing three units and one unit of antitoxin per c.c. before freezing, tested in both solutions 15 per cent. under the value of the antitoxin added, proving that even though the toxin had been heated to 80° C. for twenty minutes it still retained some neutralizing value. After freezing and thawing, both solutions had precipitates; these solutions were shaken and placed in the refrigerator to allow the precipitates to settle. The next day, a small amount of the clear solutions was removed and the remainder shaken. Both the clear and the turbid solutions of each dilution were tested.

The three units per cc. heated toxin solution showed a loss of about 30 per cent. of the antitoxin and the one unit solution 25 per cent. loss through freezing. The clear and turbid solutions of both dilutions were of the same value, proving that the precipitate was not antitoxin. Whether the freezing caused a direct actual destruction or caused a further combination of the antitoxin with the heated toxin we can not state at the present time. We are inclined to believe it is a further combination, as the freezing of the antitoxin diluted to the same value with saline solution suffered no loss. This work was repeated with another heated toxin with like results.

A diphtheria toxin was diluted with two parts of saline solution and frozen. Before freezing the strength was 0.033 and after freezing, tests showed it lost 90 per cent. of its strength. Three weeks after the eleven-day freezing at 16° below zero, Fahrenheit, the Massachusetts 1 L + mixture No. 101c, on retests showed that the acquired toxicity after freezing had been lost and that it now tested as it had before freezing. Some of the one cc. pigs showed definite symptoms of paralysis and four of the one cc. pigs died from 17 to 29 days from complete paralysis. The five cc. pigs died in 9 to 18 days.

Two more 0.1 L + mixtures were frozen, Nos. 100 and 104. No. 100 was prepared and tested on February 15, 1924. The one cc. pigs died from complete paralysis in 18 to 21 days; the three cc. pigs died in six and a half to seven and a half days, and the five cc. in three and a half to four and a half days. No. 104 was prepared and tested on March 13, 1924. The one cc. pigs died in 12 to 18 days and the three cc. pigs died in four to five days. The five cc. in three and a half days.

After freezing, the five cc. pigs had lost very little weight after five days. This last freezing was done April 10 and the tests after freezing were made April 11. Sufficient time had elapsed to show that these two most recent preparations had lost considerable toxicity. As it was our desire to have correct p.H. readings made on all the products which we froze in order to ascertain if the reaction of the various toxin-antitoxin mixtures varied to any great extent we broached the subject to Dr. Roland Freeman, Jr. and he in turn enlisted the services of Dr. Frances Krasnow of the Department of Biological Chemistry at Columbia University, and it is she to whom we are greatly indebted for the following readings:

Date of preparation	Product No.	pH before freezing	pH after freezing 18 hrs.	**pH after freezing 24 hours	**pH after freezing 11 days
11-9-23	0.1 L + No. 92	7.3			7.3
11-23-23	0.1 L + No. 96	7.3	7.3		
12-8-23	0.1 L + No. 98	7.3			
2-15-24	0.1 L + No. 100	7.3	7.3		
2-28-24	0.1 L + No. 103	7.3			
3-13-24	0.1 L + No. 104	7.3	7.3		
11-23-22	0.5 L + No. 57	7.3	7.3		
11-23-22	1 L + No. 58	7.3	7.3		
2-11-24	1 L + "Special"	7.5		7.5	7.5
	Albany				
	1 L + No. 91	7.6		7.6	7.6
	Mass.				
	1 L + No. 101 C	7.9		7.9	7.9

*Indicator phenolsulphonephthalein.
**Frozen 16° below zero Fahrenheit.

Whether or not these pH values have any significance we cannot state at the present time. On analysis, however, there seems to be an indication that they do. The Massachusetts 1 L + mixture No. 101c gave a pH reading of 7.9 and the toxicity was increased after freezing.

The Albany mixture has a reading of 7.6 and though this mixture was less toxic after freezing it did not entirely lose its toxicity; we failed to learn its date of preparation. This mixture, although sufficiently toxic for immunizing, was less toxic before freezing than any of the recent mixtures we tested before freezing, and although it lost some of its toxicity after freezing the loss was not as great as those of ours that were more toxic.

It tested on February 11 as follows: Two one cc. pigs did in 39 days. These pigs showed late definite paralysis. One five cc. pig died in 14 days; the other in 24 days from complete paralysis. After freezing, the five cc. doses showed some paralysis with recovery.

Next in order is our 1 L + prepared specially for this freezing work on February 11 with 0.8 of a unit per L +. This has a reading of 7.5 and although after freezing it lost considerable of its toxicity the five cc. pigs died from complete paralysis. Comparing the toxicity of this freshly prepared 1 L + and the 1 L + No. 58 on their most recent tests we have the following.

Prepared	1 L + "Special"	1 cc. doses dead 7 days
February	Tested Feb. 11, '24	5 cc. doses dead 2 and 3 days
11, 1924	Retests March 26th	5 cc. doses dead 3 and 3½ days
Prepared	1 L + No. 58	
November	Tested March 19th, '24	
1922		1 cc. doses dead 4½ and 5½ days
		5 cc. doses dead 2½ and 2½ days

After freezing, the special 1 L + caused with five cc. doses complete paralysis with death in 25 and 27 days. Whereas, 1 L + No. 58 after freezing, failed to cause any evidence of paralysis in five cc. doses.

In conclusion we suggest that for the present our pH value of 7.3 phenol sulphonephthalein indicator be taken for all preparations of toxin-antitoxin mixtures. We also suggest that whenever a new lot of aged toxin is required for preparing mixtures, samples of the first mixture should be frozen and tested.

PREPARATION OF MIXTURE OF DIPHTHERIA TOXIN-ANTITOXIN

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By Edwin J. Banzhaf and Charles K. Greenwald

In preparing the toxin-antitoxin mixture used for active immunization against diphtheria, it is essential to have diphtheria toxin of very high potency. The toxin we employ has a minimal fatal dose† of 0.002 cc. to 0.0007 cc. The advantage of using such highly potent toxins is that they may be diluted with salt solution to the toxin strength actually required, lessening in this way the concentration of other substances (meat extractives, pepton, bacillary substance, etc.), which give rise to local and constitutional reactions.

The most important factor in these reactions is the bacillary substance. The presence of this substance is due to the autolysis of the various bacteria which develop during the preliminary process of fermenting the meat infusion, as well as autolysis of the diphtheria bacillus which is grown on the finished medium for the production of toxin. Now, at Dr. Park's suggestion, the chopped meat is boiled with the required amount of water, thereby producing a sterile infusion. It is then inoculated with B. coli, which produces the necessary fermentation. By this procedure the autolytic products are reduced to only those produced in the growth of B. coli and of B. diphtheriae.

The bacillary substance cannot be eliminated by any method at present available. At best, we can only minimize the reactions in those who are hypersensitive to these substances by using only highly potent toxins which, as stated above, will allow of dilution. Other substances which are present in the broth and which contribute to the reactions disappear to a large extent during the period of storage (aging) of the toxin.

The toxin when freshly prepared, is unstable, and deterioration takes place, the rate of deterioration being relatively rapid at first but gradually becoming slower and slower. After six months or longer the toxin approaches a relative stability and is now used for the preparation of the toxin-antitoxin mixture.

Several lots of such toxin are mixed to give a total of about 60 litres. The L plus dose is then determined. The L plus dose is that amount of toxin which, when added to one unit of antitoxin, will give a mixture which injected into a 250 gram guinea pig will cause death on the fourth day. The toxins used contain from four to 6 L plus doses per cc.

As the convenient immunizing dose was set at one cc. for each injection, it was desirable to have all the preparations of toxin-antitoxin mixtures standardized to contain the same immunizing value; we therefore first set the standard at 3 L plus per cc. but later changed it to 0.1 L plus. Each mixture of toxin is consequently diluted with sterile salt solution, containing the required amount of preservative, to the above mentioned standard.

An aged standardized antitoxin is then added to the toxin, one unit of antitoxin for each L plus. The mixture therefore is theoretically toxic as is evident from the definition of the L plus dose given. The use of this proportion insures against a possible over-neutralization. If the mixture is too toxic, more antitoxin can always be added; whereas at present we would discourage the addition of toxin to adjust an over-neutralized mixture.

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† The smallest amount which will kill a 250 gram guinea pig in four days.

The calculated amount of antitoxin to be used is greatly diluted and added slowly, under aseptic precautions, to the toxin which is constantly agitated to insure a complete mixture. This is then allowed to stand for three hours before the preliminary tests are made.

For the preliminary test two groups of guinea pigs are employed. To each of one group is given one cc.; to each of the second, five cc. The injections are made subcutaneously. If the mixture is properly adjusted at this stage, the pigs receiving five cc. will die acutely, whereas those receiving one cc. will show no immediate effect but will develop paralysis after two weeks.

If the mixture is too toxic, more antitoxin is added till the above condition is reached.

The mixture is now filtered through a Berkefeld filter and stored in the refrigerator for stabilizing. During this period the toxicity of the mixture decreases probably through further combination between the toxin and antitoxin and through some deterioration of the toxin itself.

The mixture is now retested using three groups of guinea pigs, injecting one cc., two cc. and five cc. respectively.

The one cc. group should show some local edema at the site of injection and some loss of weight but should not die. The two cc. group will develop paralysis after about 18 days, dying on the twenty-first or twenty-second day. The five cc. group will develop paralysis after about 15 days and die two or three days later.

If these results are obtained, the mixture is ready for use. If not, further adjustments become necessary. Such a mixture retains practically its full immunizing power for at least one year.

As is evident from the above, there is a tendency to a change in the balance between the toxin and antitoxin during storage. Is there a possibility that dissociation ever takes place, that is, that the mixture may become acutely toxic, and therefore dangerous for human use? All our knowledge concerning the combination of toxin and antitoxin speaks against such a possibility. We have retested our toxin-antitoxin mixtures, at first every month, later every two months, carrying such retests over a period of two years and never noticed any increase in toxicity. In fact, the tendency is the reverse, that is, a loss of toxicity varying in degree with different mixtures. As a rule, the power to produce paralysis persists if the mixture is correctly balanced in the beginning.

OBSERVATIONS ON DIPHTHERIA TOXOID AS AN IMMUNIZING AGENT

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W. H. Park, E. J. Banzhaf, A. Zingher and M. C. Schroeder

Both diphtheria toxoid and diphtheria toxin-antitoxin derive their immunizing potency from altered diphtheria toxin. The freshly prepared toxin has such an instant action on the human tissues that only minute amounts can be used, and immunization with it is therefore a long drawn out process.

Diphtheria toxoid was used for animal immunization in the first experimental work in producing antitoxin and it has continued to be used to some degree ever since.

The utilization of a toxin-antitoxin mixture by von Behring led to the use by us and others of toxin-antitoxin rather than toxoid. During the past three years Glenny and Hopkins in London and Ramon in Paris have been suggesting the substitution of toxoid for toxin-antitoxin. Independently of them we had begun to test the value of toxoid preparations.

Diphtheria toxin can be changed to toxoid by various means, such as storage at moderate temperatures, heating to 50 to 54° C. for a few hours, or exposure to chemicals.

The accepted method at the present time is to add 0.2 to 0.3 per cent. of formalin to the toxin, the amount depending on the protein content and the duration of storage in the incubator.

The advantages claimed for the toxoid are that it is simpler to prepare; is more stable; is somewhat more potent, and it contains no horse serum globulin. In our opinion toxoid is no easier to prepare than toxin-antitoxin. Experience and the following out carefully of every detail of a proper method are necessary in both cases. To the inexperienced a toxoid is easier to prepare because it is a single substance instead of two substances and the difficulty of making a proper mixture is avoided.

The question of stability is more debatable. The longer a toxin is stored, and to a certain extent the greater the percentage of change from toxin to toxoid, the less rapid will be any further change.

The toxin used for toxin-antitoxin is really a mixture of toxin and toxoid. If the toxin-antitoxin mixture is made so that when mixed it has just the right toxicity, it will drop off in potency faster than a toxoid preparation, but if it is made with some excess of toxicity and stored for several months before it becomes suitable for use it will then remain as stable or nearly as stable as the toxoid preparations.

The objection that toxin-antitoxin has a minute amount of horse serum globulin, and as shown by Hooker suffices to cause a moderate serum sensitization, has some weight. It can be obviated by following the suggestion of Hooker of using goat antitoxin. Banzhaf has already prepared such a prepration, and this has been used in a number of children by Schroeder and Zingher. Re-tests have not yet been made. We have never seen any dangerous reactions following the use of serum in those who previously had toxin-antitoxin. We feel certain that there is no objection to giving previously injected children an intramuscular injection of serum. We need more experience before being as sure as to the effect of this slight degree of sensitization on the reaction to an intravenous dose. There is a tendency to attempt to use a toxoid or a toxin-antitoxin preparation that has no toxic effect on guinea pigs. This is very effective in animals, but has a decided drawback. Thus the toxoid sent us by Dr. O'Brien from the Burroughs Wellcome laboratory can be given in five cc. doses to guinea pigs, and has remarkable immunizing potency in this dose. In man, however, one-half a cc. is advisable because the pseudo reaction to the bacillus proteins makes too severe a local reaction. It practically is the same as the old three or five L plus preparations. The comparative reactions from one cc. and from one half cc. of the nontoxic toxoid and one cc. of the 0.1 L plus toxin-antitoxin are shown in Table II. Glenny and Hopkins and Ramon believe that the amount of absorption of antitoxin by a preparation is a pretty exact index of its immunizing power.

This may be true as between different preparations made in the same dilutions from the same materials, and as far as possible in the same manner, but it is far from true if used to test preparations made in different ways and of different degrees of toxicity. The larger the amount of the original toxin in the human dose, the greater will be its antitoxic neutralizing value.

Table I shows the relative absorption of antitoxin by a human dose and the relative potency of different toxoid preparations, as judged by the immunizing results in guinea pigs of a single injection followed by a stimulating

TABLE I.

Preparation	Human Dose	Units of Antitoxin bound by the test dose	Toxicity in 250 gram guinea pigs	Human Doses used as test injections in guinea pigs	After one dose and one Schick test percentage of 15 guinea pigs giving a negative Schick reaction at end of four weeks
B. Wellcome Toxoid 1923	0.5 cc.	3.5	5 c.c. no effect	2.0	70
Same 8 mos. later	0.5 cc.	3.5	5 c.c. no effect	2.0	60
B. Wellcome Toxoid 1924 preparation	0.5 cc.	3.5	5 c.c. no effect	1.0	35
Banzhaf 0.25% formalin					
Toxoid 437	0.5 cc.	2.5	5 c.c. sl. paralysis	2.0	76
Toxoid 350	0.5 cc.	2.5	5 c.c. died 18 days	2.0	70
Banzhaf 0.1% formalin					
Toxoid 437Z	0.1 cc.	0.37	0.2 c.c. sl. paralysis	1.0	15
Toxoid 377Z	0.1 cc.	0.20	0.2 c.c. sl. paralysis	1.0	50
Banzhaf heated 54°C. 5 hrs. Toxoid			5 c.c. no effect	1.0	24
Toxin-antitoxin as comparison					
T.A. 110 0.1L plus			1 c.c. paralysis 21 das.	0.5	85
T.A. 69 0.1L plus			5 c.c. paralysis 27 das.	1.0	65
T.A. 86 0.1L plus			1 c.c. paralysis and recovery	0.5	10
T. A. Solution of precipitate from mixing Toxin and Antitoxin					
Precipitate of toxin 377 Banzhaf			1 c.c. kills 30 days	0.2	80
Precipitate of toxin 517 Banzhaf			1 c.c. slight paralysis	1.0	100

Schick test at the end of the third week and a final Schick test at the end of the fourth week. A completely negative Schick test is considered as indicating immunity.

The results of these tests indicate that the best toxoid preparations we have at present are nearly, but not quite, as effective as the best toxin-anitoxin preparations. If only the non-specific proteins could be removed, larger doses of toxoid could be used, and even better results obtained.

RESULTS OF IMMUNIZATION OF MAN

One cc. of original toxoid 377 diluted 1 in 14 to 1 in 20, given to 659 children originally Schick positive, when re-tested by Zingher four to six months afterwards gave 448 negative reactions, or 68 per cent. effective results. Schroeder gave three injections of the same preparation to 187 adults with 87 per cent. apparent success. As control tests were not used by Schroeder with the first injections this should be reduced to about 80 per cent.

With toxoid 437, Zingher got better results—1,525 children giving a positive reaction received three injections of one cc. of a 1 to 20 dilution of the original toxoid. At the re-test two and one-half to three months later, 1,342, or 8 per cent. gave a negative reaction.

Comparing the results we find that toxoid 437, containing a larger amount of antitoxin neutralizing substance, gave the better results in man, but the force of this observation is lessened by the fact that in the test on guinea pigs the reverse result was obtained.

The local and constitutional effects of the diluted slightly toxic toxoid as noted by Zingher and Schroeder were very similar to those following the standard 1|10 L plus preparation of toxin-antitoxin of the same toxicity.

The reaction following the use of non-toxic toxoid was wholly due to the proteins, as there was no active toxin present. In Table II a comparison between the Burroughs and Wellcome toxoid and toxin-antitoxin is given.

TABLE II.

COMPARISON BETWEEN THE REACTIONS DUE TO THE SUBCUTANEOUS INJECTIONS OF THE NONTOXIC TOXOID (BURROUGHS WELLCOME & CO.) AND THE STANDARD 1/10 L PLUS TOXIN-ANTITOXIN IN CHILDREN (SCHROEDER).

29 children (Schick + 10 Schick — 19) receiving 1 cc. of toxoid. Subcutaneous reaction + 16; ± 9; ∓ 3; — 1.

23 children (Schick + 9 Schick — 14) receiving 0.5 cc. of toxoid. Subcutaneous reaction + 5: + 12: ∓ 3: — 3.

29 children (Schick + 5 Schick — 24) receiving 1 cc. standard 1/10 L plus toxin-antitoxin. Reaction + 6; ± 6; ∓ 4; — 13.

Average diameter of the 1 cc. toxoid reaction 5 cm., ½ cc. toxoid 1.7 cm. 1 cc. T-A 1.7 cm. One cc. of undiluted toxoid gives a larger percentage of pseudo-reactions and more intense reactions than the 1/10 L plus T. A. The ½ c.c. dose of toxoid gives a greater number of marked pseudo-reactions but of no greater intensity than the 1/0 plus T.A.

SUMMARY

At the present time toxoid preparations have been developed which are nearly, if not quite, equal to the best toxin-antitoxin preparations. The toxoid preparations which have no toxicity in guinea pigs cannot be properly used in human beings in doses larger than one-half c.c. because of the marked pseudo-reactions. Toxoid that is slightly toxic can be diluted at least ten times and so reduce the amount of proteins. It is a question whether the slightly toxic diluted toxoid preparations are as stable as the nontoxic ones. Intensive experimental work should be done in both toxoid and toxin-antitoxin preparations so as to be able constantly to have thoroughly potent and stable preparations. After one or two years we should be able to decide which has the greater advantages.

For bibliography see the original article.

THE GRADUAL DECREASE OF THE ACTIVE IMMUNIZING VALUE OF TOXIN-ANTITOXIN MIXTURES STORED DURING ONE YEAR

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Edwin J. Banzhaf

About two years ago when the active immunization from the 3 and 5 L + toxin-antitoxin mixtures were compared the results showed that the 5 L + did not give any greater percentage of active immunity in children than the 3 L +. The 5 L + however did give a higher percentage of local and constitutional reactions in children and adults. These were due to the greater amount of protein substances in the mixture. The 5 L + mixtures were made with the aged diphtheria toxin without any dilution other than that due to the addition of the proper amount of antitoxin, whereas the 3 L + mixtures were prepared from the 5 L + toxin, diluted with water to contain 3 L + and therefore had 40 per cent. less of the reacting substances. Even with this lesser amount of reacting substances, the 3 L + mixtures gave annoying reactions. Every known method had been tried to eliminate these substances, but without success. Knowing that the 5 L + did not give a greater percentage of active immunity than the then standard 3 L +, Dr. Park suggested, after testing several different preparations, a 0.1 L + mixture. That is a mixture that would contain only 3⅓ per cent. of the reacting protein substances of the standard 3 L + and would be neutralized with antitoxin so that the mixture would react in guinea pigs exactly like the 3 L + mixtures. The first experimental 0.1 L + mixture was prepared in the early spring of 1922 by diluting the aged toxin with tap water containing 0.8 per cent. sodium chloride and 0.4 per cent. trikresol. Three hours later 0.5 unit of antitoxin was added to each L + and thoroughly mixed, Berkefelded and tested. Two out of three guinea pigs which received one cc. doses died of late paralysis. The third recovered. One five cc. pig died within five days. The reactions in humans with this mixture were in the majority practically nil. The immunity response was equal to that given by the 3 and 5 L + mixtures, and the response in the children was equal to that in the guinea pigs.

Several months later, on retesting the experimental mixture, it was found to be less stable than the standard 3 L + and that it had lost a considerable amount of its immunizing value. Five months after it was prepared guinea pigs showed the following: three cubic centimeters slight symptoms of paralysis; five cubic centimeters paralysis with death in thirty-seven days. Three months later, five cc. gave slight symptoms of paralysis and ten cc. gave definite symptoms with recovery.

This was its only drawback. In order to overcome it considerable work was done to evolve a method of preparing the mixtures in such manner that they would retain their immunizing value at least nine months.

The immunizing values of two preparations made from the same lot of aged toxin which were mixed by different methods were followed for thirteen months.

Preparation 67 was purposely made very toxic and was prepared February 30, 1922, as follows: The required amount of 0.8 per cent. salt diluent containing 0.4 per cent. alcresta was measured into a suitable container. The required amount of toxin was added to the diluent and thoroughly mixed. The desired amount of antitoxin, in this preparation 0.75 unit per L +, was added immediately to the diluted toxin, thoroughly mixed, Berkefelded and tested. The one cc. pigs died within six to nine days and the five cc. within two to two and one-half days. This proved more toxic than intended. To hasten the decrease of toxicity an 8-liter stock bottle of the preparation was kept at room temperature for two weeks and labeled 67a. Tests were put on November 27, 1922. The one cc. pigs developed paralysis and died within twenty to twenty-six days. The five cc. pigs died within two and one-half days.

The second preparation 69 was intended for immediate use and was prepared on November 15, 1922 as follows: The same amount of the same toxin as used for preparation 67 was measured into a liter bottle and the desired amount of antitoxin, in this case 0.8 of a unit per L+, was measured into the liter bottle containing the toxin, the bottle stoppered and thoroughly shaken. The same amount of salt diluent containing 0.5 per cent. phenol as was used in preparation 67 was measured into a suitable container. The concentrated toxin-antitoxin was then added to the diluent with constant stirring. The bottle contents and the diluent were thoroughly mixed and Berkefelded on the same day. Tested on November 22, 1922, the one cc. pigs died from paralysis within twenty-two days. The three cc. pigs within ten and nineteen days and the five cc. within three and one-half to four and one-half days.

Immunization, in guinea pigs were started with these two preparations on December 5, 1922. The average weight of the pigs was 275 grams.

The injections were given subcutaneously as follows:

PREPARATION NUMBER	NUMBER OF GUINEA-PIGS	AMOUNT INJECTED	WEEKLY INJECTIONS
		cc.	
67a	12	0.1	3
67a	12	0.2	3
69	12	0.1	3
69	12	0.2	3

A like series was injected every six to ten weeks with the exception of the last where the interval was thirteen weeks. Eleven to twelve weeks after the first injection, these pigs were Schicked for immunity value.

For readings of partial immunity in those pigs which might not show a negative reading when given the standard dose of 1/50 of an M.L.D. in 0.1 cc. weaker doses were also given.

Each pig received 1/150, 1/100 and 1/50 of an M.L.D. intracutaneously. The final readings were taken four days later. The readings were recorded as follows:

1/150	1/100	1/50	
+ —	+	+	Normal guinea-pig will die with 1 M.L.D.
+ — —	+ —	+	
+ — — —	+ — —	+ —	Slight immunity will survive 1 M.L.D.
—	+ — — —	+ — —	Partial immunity will survive 1½ M.L.D.
—	—	—	Immunized will survive 2 or more M.L.D.

The figures in the following table are given in percentages:

DATE TESTED	PREPARATION 67a				PREPARATION 69			
	Amount	Paralysis	Number of days for death	Remarks	Amount	Paralysis	Number of days for death	Remarks
	cc.				cc.			
November 27, 1922	1	+	20		1	+	22	
	1	+	26		1	+	22	
	5		2½		3		10	
	5		2½		3	+	19	
					5		3½	
					5		4½	
December 27, 1922	1	+	28		1	+	28	
	1		13		1	+		Recovered
	1	+	18		3	+	16	
	1	+	21		3	+	15	
	3		7		5		12	
	3		6		5		5	
	5		3					
	5		3					
April 4, 1923	1	(?)		Recovered	1	(?)		Recovered
	1	(?)		Recovered	1	+	29	
	3	+	25		3	+	21	
	3	+	29		3	+	29	
	5		12		5	+	18	
	5	+	18		5	+	16	
July 13, 1923	1	(?)		Recovered	1	+		Recovered
	1	(?)		Recovered	1	+		Recovered
	3	+		Recovered	3	+	26	Recovered
	3	+		Recovered	3	+	26	
	5	+	25		5	+	15	
	5	+	24		5	+	18	
November 23, 1923	3	(?)		Recovered	3	+		Recovered
	3	(?)		Recovered	3	+		Recovered
	5	+		Recovered	5	+	27	
	5	+	34		5	+	27	

During the time the preparation showed on retesting sufficient toxicity to kill the five cc. pigs acutely, it was considered advisable to Schick earlier than after twelve weeks, in order to catch some of the pigs before evidences of immunity could be detected. The first series was Schicked after eleven and one-half weeks and not one of the series gave the normal test.

The second series were Schicked after eleven weeks and in this all stages were caught from still normal up to complete immunity. As both preparations were showing decreased toxicity the third series was Schicked after eleven and one-half weeks. There is considerably more immunity developed after eleven and one-half and twelve weeks than after eleven and this explains the higher percentage of immunity of series three and four over the second series which was Schicked after eleven weeks. From the fourth series on, the elapsed time for the Schicking was constant and shows the gradual decrease in the percentage of those immunized. In the last series, preparation 67a shows a sharp drop in the per cent. of those completely immunized. This was expected as can be seen in a table in the original article on the retesting of the preparations. The retest on November 23, 1923, showed only questionable evidences of paralysis in those pigs which received three cc. and paralysis with recovery in one of the five cc. pigs.

Preparation 69 retested on the same date still caused definite paralysis in three cc. and death with paralysis with five cc. doses.

From the work reported and other unfinished work it can be said that toxin-antitoxin mixtures which cause paralysis in guinea pigs with three cc. doses and paralysis with death in five cc. doses is still toxic enough for immunizing, and those mixtures which fail to cause death through paralysis with five cc. doses are too weak. Although 67a was more toxic than 69 when the immunization started, it did not retain the toxicity as long as 69. It cannot be stated at this time that the method of mixing preparation 69 is superior to that of mixing 67 in as far as retaining its toxicity is concerned. It is superior, though, in that it takes less time, less handling, and its accurateness can be more easily controlled. Since preparation 69 all our mixtures have been prepared in like manner and are in like manner holding much of their value for at least a year.

PREPARATION NUMBER	THREE DOSES	SCHICK TEST READING (1/150, 1/100, 1/50)			DATE OF FIRST INJECTION DECEMBER 5, 1922; SCHICKED AFTER ELEVEN AND ONE-HALF WEEKS	DATE OF FIRST INJECTION JANUARY 20, 1923; SCHICKED AFTER ELEVEN WEEKS	DATE OF FIRST INJECTION MARCH 3, 1923; SCHICKED AFTER ELEVEN AND ONE-HALF WEEKS	DATE OF FIRST INJECTION MAY 27, 1923; SCHICKED AFTER TWELVE WEEKS	DATE OF FIRST INJECTION JULY 6 1923; SCHICKED AFTER TWELVE WEEKS	DATE OF FIRST INJECTION SEPTEMBER 6, 1923; SCHICKED AFTER TWELVE WEEKS	DATE OF FIRST INJECTION DECEMBER 14, 1923; SCHICKED AFTER TWELVE WEEKS
67a	cc. 0.1	—	—	—	78	25	50	30	27	27	10
	0.2	—	—	—	86	58	58	70	66	63	9
	0.1	—	+ — — —	+ — —	11	25	16	10	18	9	20
	0.2	— —	+ — — —	+ —	14	8	8	10	9	1	27
	0.1	+ — — —	+ — —	+	11	42	8	30	28	18	30
	0.2	+ — — —	+ — —	+ —	0	16	25	0	8	18	18
	0.1	+ — — + —	+ — +	+ +	0	8	34	30	27	45	40
	0.2	+ — — + —	+ — +	+	0	16	8	20	16	18	45

PREPARATION NUMBER	THREE DOSES	SCHICK TEST READING (1/150, 1/100, 1/50)			DATE OF FIRST INJECTION DECEMBER 5, 1922; SCHICKED AFTER ELEVEN AND ONE-HALF WEEKS	DATE OF FIRST INJECTION JANUARY 20, 1923; SCHICKED AFTER ELEVEN WEEKS	DATE OF FIRST INJECTION MARCH 3, 1923; SCHICKED AFTER ELEVEN AND ONE-HALF WEEKS	DATE OF FIRST INJECTION MAY 27, 1923; SCHICKED AFTER TWELVE WEEKS	DATE OF FIRST INJECTION JULY 6, 1923; SCHICKED AFTER TWELVE WEEKS	DATE OF FIRST INJECTION SEPTEMBER 6, 1923; SCHICKED AFTER TWELVE WEEKS	DATE OF FIRST INJECTION DECEMBER 14, 1923; SCHICKED AFTER TWELVE WEEKS
69	cc. 0.1	—	—	—	75	18	75	34	30	25	18
	0.2	—	—	—	88	42	70	82	62	54	42
	0.1	—	+ — — —	+ — —	13	27	0	16	20	25	27
	0.2	—	+ — — —	+ — —	0	8	10	18	12	18	25
	0.1	+ — — —	+ — —	+ —	12	18	16	0	30	34	36
	0.2	+ — — —	+ — —	+ —	12	42	20	0	25	18	16
	0.1	+ — —	+ —	+	0	36	8	50	20	16	18
		+ —	+	+							
	0.2	+ — —	+ —	+	0	8	0	0	0	10	16
		+ —	+	+							

THE PREPARATION OF 0.1 L + TOXIN ANTITOXIN MIXTURE

Edwin J. Banzhaf

The media for Diphtheria toxin to be utilized for toxin antitoxin mixtures is prepared in the usual way with the following exceptions:

To every pound of ground fat-free veal, a liter of water is added and placed in a refrigerator over night. The following morning the liquid is collected and boiled. The media is boiled at this stage to kill all organisms except the few heat-resisting spores, thereby practically limiting the bacterial protein soluble substances to the coli and diphtheria bacilli with which it is inoculated later. After boiling, the reaction is corrected to 1.5 per cent. acid to phenolphthalein; 10 cc. of a 24 hour culture of B. coli is added for every liter of media and incubated about 18 hours. One egg for every two liters is used, then boiled and strained. The reaction is corrected to 0.5 per cent. acid to phenolphthalein. One per cent. of peptone is added (Parke, Davis and Company) and 0.5 per cent. sodium chloride. The reaction is again corrected to 0.5, the media filtered into flasks and autoclaved. At the time of planting one per cent. of glucose is placed in each flask.

The diphtheria toxin is prepared in lots of about 16 liters and stored in gallon bottles for at least a year. When wanted for the preparation of toxin-antitoxin mixture a lot is poured into a suitable sterile container and thoroughly mixed and again poured into the gallon bottles, thereby insuring its uniformity. These bottles are properly labelled and numbered and the L+ determined.

Three preliminary toxin-antitoxin mixtures are made, to establish the exact amount of antitoxin to be added to each L+, also to determine whether the toxin lot has, in sufficient amounts, that portion of toxin which causes paralysis. The three preliminary mixtures of eight liters each, containing 0.1 L + are prepared and Berkefelded. These contain the following amounts of antitoxin: the first 0.75 of a unit to each 1 +, the second, 0.77 unit and the third 0.8 unit. The mixture which causes death in from five to ten days with five cc. doses in 300 gram guinea pigs and paralysis with death with one cc. doses in 250 to 260 gram pigs has the proper amount of antitoxin added to each L +. If the five cc. dose pigs die as stated and the one cc. dose pigs show no definite symptoms of paralysis the lot of toxin is considered too low in that portion of toxin which causes paralysis and is unsuitable for toxin-antitoxin mixtures. These three preliminary mixtures are frozen at minus 8° C. for 18 hours. They are then thawed at room temperature and retested for any change in toxicity. We have always found them to be less toxic after freezing. No further freezing tests are necessary for this subsequent preparation made with the same stock lot of toxin.

When the proper amount of antitoxin to be added to the stock toxin is found, subsequent mixtures of multiple amounts of each will give the same desired results providing the same measuring apparatus and the same procedure is followed.

The procedure: The required amount of toxin is accurately measured into a sterile pint bottle. The antitoxin is diluted so that each one cc. contains 20 units. The required amount is accurately measured into a pint bottle containing the toxin. The bottle is stoppered and thoroughly shaken and placed in a refrigerator until the diluent (sterile physiological salt solution containing 0.5% phenol) has been measured into a suitable sterile container. The concentrated toxin-antitoxin mixture is then added with constant stirring and the bottle rinsed with the diluent.

Although our stock lots of toxin have shown no tendency to deteriorate to any extent in their L + value or noticeably to lose that portion of toxin which causes paralysis, we always standardize two stock lots of aged toxin for our mixtures.

In preparing the mixtures we prepare two: one from each lot of stock toxin. The two lots of stock toxin containing about 16 liters each are sufficient for about a year's supply of 0.1 L + toxin antitoxin mixture. After determining the L + value of the lots of stock toxin and establishing the proper amount of antitoxin to be added to the L + no further standardizing is necessary. If in the three preliminary toxin-antitoxin mixtures the results showed that 0.8 of a unit was the proper amount to add to each L + and four to six months later mixtures prepared with this amount prolonged the life of all the guinea pigs receiving five cc. doses beyond the five-day period we would have the mixture used immediately. The next mixture would then be prepared with 0.75 of a unit per L +. Should this mixture prove too toxic it would be stored in a refrigerator until retests proved it ready for use. No attempt is ever made to add antitoxin to an excessively toxic mixture. Another mixture would then be prepared with 0.77 or 0.78 of a unit.

Preparations of mixtures are made in 32 liter lots. The three agate cans of 48 quart capacity each, which are used only for this product, are sterilized by placing into each about 18 liters of a five per cent. phenol solution, the covers adjusted and the whole boiled for 30 minutes. The phenol solution is poured from two of the cans and 35 liters of water containing 0.8% salt added to each can, the covers adjusted and again boiled for one hour. After cooling, 0.5 per cent. phenol is added and the cans placed in a refrigerator over night during the summer months. The following morning the required amounts of the two standardized stock lots of toxin are placed in two sterile one-liter bottles and immediately marked with their proper respective preparation number. The required amount of antitoxin is added to each, and each bottle is then stoppered and shaken. They are put into a refrigerator while the diluents are measured.

The third can containing the phenol solution is emptied and the required amount of the diluent salt solution measured into it and immediately marked with the preparation number, corresponding to its concentrated toxin-antitoxin mixture. The remaining excess of salt solution is discarded and into that can the second amount of diluent is measured and marked with its preparation number. The excess diluent in the third can is now discarded. This can is utilized for the mixing. The concentrated toxin-antitoxin mixture is now added to the diluent corresponding to its number, with constant stirring, and the bottle rinsed with the diluent. As soon as added the mixture is immediately poured into the empty third can and then back again into the original. This is repeated four times. The second bottle of concentrated toxin-antitoxin is now added to its diluent and mixed by pouring in the same manner. Both preparations are immediately filtered through the Berkefeld and tested on the same day. The temperature of the diluent should never be above 22° C.

The time required to prepare, mix and Berkefeld two preparations of 32 liters each, not including the time to sterilize the cans and salt diluent, is less than three hours.

The pH. reaction of the mixtures is adjusted to 7.3 using Phenol Sulphophthalin as the indicator.

THE EFFECT OF PEPTON UPON THE PRODUCTION OF TETANUS TOXIN

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Harriet Leslie Wilcox

Not very long after the outbreak of the European War, laboratory workers realized that a substitute for Witte pepton in bacteriological work would have to be obtained. Many laboratories were sufficiently stocked with this pepton for the first twelve to eighteen months, but in the spring of 1915 it was evident that the supply of Witte pepton in the United States was fast dwindling and that it would soon be unobtainable.

Witte Pepton

At the Bureau of Laboratories we had been using a Witte pepton glucose veal broth with extremely satisfactory results. Those who have had experience in toxin production, particularly tetanus toxin, know that from time to time there will be marked variation in toxicity due to some unknown factor or factors. The same care may be used in the preparation of different lots of broth as well as in the filtration of the toxin, but the results may differ widely. In consideration of this fact it may be worth while to give the different potencies of the toxin made during the last three to four years when using Witte pepton glucose veal broth.

It is the opinion of some workers that the time of year has a definite influence upon the production of potent toxin. The results as given above show that the seasonal factor was of negative importance as far as toxicity was concerned. If we compare the quarterly results for the year 1915 we find that the averages did not vary greatly. The lowest average toxicity of 1:20,000 occurred in the first, third and fourth quarters, while the second quarter gave an average toxicity of 1:27,000.

The differences in the quarterly averages of the year 1916 were so slight that they were scarcely worth mentioning. The third quarter in this period showed the lowest average toxicity of 1:20,000 while the first, second and fourth quarters gave an average toxicity of 1:22,000.

Doubtless if Witte pepton veal broth had been used throughout the year 1917 we should have had a greater variation in the quarterly averages, due to the two extremely potent toxins produced in the third quarter. As far as was known no especial care was taken in the prepartion of these two broths nor in the filtration of these toxins. In lot 127, the usual procedure of testing for 1:15,000 and 1:25,000 was carried out. When the pigs succumbed in less than forty-eight hours, higher tests, namely 1:40,000 and 1:60,000 were made with similar results. Dilutions of 1:80,000 and 1:100,000 were then injected into guinea pigs weighing 350 grams each. The pig receiving the 1:80,000 dilution died between the second and third day, while the pig which received the 1:100,000 dilution succumbed on the fourth day. Since this was the strongest toxin ever obtained at the Bureau of Laboratories, tests were made of the toxin filtrate to preclude any possibility of spores or bacilli having passed thrugh the Berkefeld filter. These tests were negative.

TABLE 1

WITTE PEPTONE

	1914	1915	1916	1917	1918
First quarter	None produced	7 lots 1:25,000 2 lots 1:15,000† 1 lot 1:10,000* 1 lot 1: 5,000 Average toxicity 1:20,000 Excluding*† average toxicity 1:22,000	1 lot 1:35,000 2 lots 1:30,000 2 lots 1:25,000 1 lot 1:20,000 2 lots 1:15,000 1 lot 1:13,000 1 lot 1:10,000§ 1 lot Below 1:5,000** Average toxicity 1:20,000 Excluding§** average toxicity 1:22,000	None produced	1 lot 1:25,000 1 lot 1:45,000 Average toxicity 1:35,000
Second quarter	None produced	5 lots 1:30,000 4 lots 1:25,000 1 lot 1:20,000 Average toxicity 1:27,000	4 lots 1:35,000 1 lot 1:10,000 1 lot 1: 5,000* Average toxicity 1:19,000 Excluding* average toxicity 1:22,000	None produced	None produced

(*Continued on next page*)

(Continued from previous page)

Third quarter	2 lots 1:25,000	1 lot 1:40,000 2 lots 1:30,000 6 lots 1:25,000 3 lots 1:20,000 3 lots 1:15,000 2 lots 1:10,000 1 lot Below 1:5,000‡ Average toxicity 1:20,000	1 lot 1:30,000 3 lots 1:25,000 1 lot 1:20,000 2 lots 1:15,000	1 lot 1:100,000†† 1 lot 1: 60,000 1 lot 1: 25,000 2 lots 1: 20,000	None produced
	Average toxicity 1:25,000	Excluding‡ average toxicity 1:22,000	Average toxicity 1:20,000	Average toxicity 1:45,000	
Fourth quarter	1 lot 1:40,000 5 lots 1:25,000	2 lots 1:35,000 5 lots 1:25,000 1 lot 1:15,000 2 lots 1:10,000 2 lots 1: 5,000	1 lot 1:25,000 1 lot 1:25,000	1 lot 1:35,000 1 lot 1:20,000	None produced
	Average toxicity 1:27,000	Average toxicity 1:20,000	Average toxicity 1:25,000	Average toxicity 1:27,000	

*Flasks contaminated.

†Temperature of incubation down to 32°C

‡Broth very dark, due probably to over heating of glucose.

§Broth very acid (2.2) after sterilization.

**Two lots of toxin were interchanged through error when removed from the incubator. Lot inoculated 1-26-16 was mixed with that of 2-2-16.

††This preparation is referred to in text as lot 127.

DIFFERENT PEPTONS

In the spring of 1916, we anticipated the present shortage of Witte pepton and made comparative tests with some of the domestic peptons on the market. These results are to be regarded as comparative only, but in those that were controlled by the use of Witte pepton, the differences in the toxicity, with one or two exceptions, were very significant.

A subsequent test, made with another preparation of Fairchild pepton, gave a toxicity of only 1:8000. No control test was made as we were unable to obtain Witte pepton.

TABLE 2.

COMPARATIVE TESTS OF DIFFERENT PEPTONS

TEST	NAME OF PEPTON	TOXICITY
1	Witte (control)	1:25,000
	Leitz	1:5,000
	Eimer and Amend	1:1,000
2	Witte (control)	1:25,000
	Squibb	1:5,000
	Research no. 2	1:15,000
3	Witte (control)	1:25,000
	Fairchild	1:20,000
	Research no. 2	1:20,000
4	Witte (control)	1:30,000
	Parke, Davis and Company	1:8,000

MARTIN PEPTON

Sporadic attempts at using Martin pepton broth were made early in the year 1916 with indifferent results. Nothing more was done until January, 1917, with regard to the adoption of Martin broth for tetanus toxin as used at the Pasteur Institute. The entire absence of Witte pepton at this time prevented us from making control tests with the usual Witte pepton glucose veal broth.

The highest average for this period, i.e., in the year 1917, occurred in the second quarter, when the average toxicity was 1:17,000. The first and third quarters gave respectively the average toxicity of 1:10,000 and 1:9000 while the fourth quarter gave the lowest average toxicity of 1:5,000.

Three different strains of *Clostridium tetani* were used for a comparative test (table 4). The strain designated "Research" is the culture used in the routine production of tetanus toxin at the Bureau of Laboratories. This strain was obtained about ten years ago from the New York State Laboratory at Albany but its origin and date of isolation are not known. Unless otherwise stated this was the strain used in our tests. The Goadby strain was obtained from Dr. MacConkey, Lister Institute, England, in 1916, and had been isolated from a war case by Dr. Goadby in 1915. The Pasteur strain was the one formerly in use at the Pasteur Institute, Paris, for the production of tetanus toxin. That this strain did not produce so potent a toxin (1:25,000) as the Research and Goadby strains (1:35,000) was somewhat

TABLE 3.

MARTIN PEPTON VEAL BROTH

	1917	1918
First quarter	2 lots 1:15,000 1 lot Below 1:5,000 Average toxicity 1:10,000	2 lots below 1:5,000 Average toxicity below 1:5,000
Second quarter	1 lot 1:25,000 1 lot 1:10,000 Average toxicity 1:17,000	1 lot below 1:5,000 Average toxicity below 1:5,000
Third quarter	1 lot 1:15,000 2 lots 1:8,000 1 lot 1:5,000 Average toxicity 1:9,000	None produced
Fourth quarter	1 lot 1:15,000 2 lots 1:8,000 3 lots 1:5,000 3 lots Below 1:5,000 Average toxicity 1:5,000	None produced

surprising as it had been kept on a medium made with Martin pepton for years, whereas the Goadby and Research strains had been accustomed to this medium for several months only.

That Martin pepton broth as made by us is capable occasionally of giving potent toxin, is evidenced by the results obtained in experiment 1 (table 4) and in experiment 4 (table 5.) The other preparations of toxin broth were made with the same precautions and care but, as is shown, with far different results. When it was apparent that equal parts of Martin pepton and veal

TABLE 4

COMPARATIVE TESTS OF DIFFERENT STRAINS IN MARTIN PEPTON VEAL BROTH

	Strains	Toxicity
Experiment 1. December 23, 1916	Research Goadby Pasteur	1:35,000 1:35,000 1:25,000
Experiment 2. May, 19, 1917	Research Goadby	1:10,000 1:15,000
Experiment 3. December 15, 1917	Research Pasteur Pasteur	1:8,000 1:8,000 1:8,000

infusion were not giving satisfactory toxin, tests were made with broth prepared by using more concentrated Martin pepton solution with ordinary veal infusion (experiment 4, table 5) ; also by using the usual amount of Martin pepton solution with a more highly concentrated veal infusion. In neither case was the result so satisfactory as with the usual preparation. Subsequent broths were prepared according to the original method, that is to say using equal parts of infusion and pepton, but the toxin was far too low in potency to be used.

Word was received in January, 1918, with regard to the modification of Martin pepton broth used with favorable results for tetanus toxin at the Pasteur Institute. The variation between this procedure and the one we were using lay in the concentration of the pepton solution, in the shortened incubation period and also in the absence of glucose. A preparation of toxin broth (see experiment 6, table 6), was made according to this modification, one-half of the broth having one per cent glucose added to it while the other half was prepared without any sugar. After seven days incubation instead of the usual period of fifteen days, the cultures were filtered and tested. The results of the animal tests were most disappointing as pigs inoculated with one c.c. of a dilution of 1:5,000 from each preparation showed an absence of tetanic symptoms. In his article on the preparation of this pepton, Martin advises that not less than five stomachs should be used owing to the great variation of the different stomachs. We are accustomed to make about fifteen liters of pepton solution for one preparation of toxin broth. This amount requires seven or eight stomachs according to the size. At the Pasteur Institute, marmites or large casseroles holding not less than 60 liters are employed for the digestion of the pigs' stomachs.

TABLE 5

COMPARISON OF DIFFERENT METHODS FOR THE PREPARATION OF MARTIN PEPTON VEAL BROTH

	MEDIA	STRAIN	TOXICITY
Experiment 4. June 13, 1917......	*Control broth* 1 part Martin pepton solution (200 grams of minced stomach to 1000 cc. H_2O) 1 part veal infusion (500 grams of veal to 100 cc. H_2O)	Research	1:25,000
	Experimental broth 1 part of Martin pepton solution 1 part of veal infusion (500 grams of veal to 500 cc. H_2O)	Research	1:8,000
	Experimental broth 1 part of Martin pepton solution (400 grams of stomach to 1000 cc. H_2O) 1 part of veal infusion	Research	Below 1:5,000
Experiment 5. October 10, 1917...	*Control broth* 1 part Martin pepton 1 part of veal infusion	Research	1:5,000
	Experimental broth 1 part of Martin pepton solution 2 parts of veal infusion	Research	1:8,000
	Experimental broth 1 part of Martin pepton solution (200 grams stomach to 1500 cc. H_2O) 1 part of veal infusion	Research	1:5,000
Experiment 6. April 2, 1918......	1 part of Martin pepton solution (300 grams of stomach instead of usual 200 grams to 1000 cc. H_2O)	Research	
	1 part of veal infusion To the above mixture of pepton and veal infusion 1 per cent glucose was added	Research	Below 1:5,000
	1 part of Martin pepton (300 grams of stomach to 1000 cc. H_2O) 1 part of veal infusion. No glucose	Research	Below 1:5,000

TABLE 6

BERNA PEPTON

	1918	1919	1920
First quarter	None produced	1 lot 1:40,000 1 lot 1:35,000 1 lot 1:30,000 1 lot 1:25,000 1 lot 1:20,000 5 lots 1:15,000 4 lots 1:10.000 4 lots 1:8,000 1 lot 1:4,000 Average toxicity 1:15,000	1 lot 1:35,000 1 lot 1:30,000 1 lot 1:15,000 1 lot 1:10,000 Average toxicity 1:22,000
Second quarter ...	1 lot 1:100,000 1 lot 1: 45,000 Average toxicity 1:72,000	1 lot 1:40,000 4 lots 1:35,000 1 lot 1:30,000 4 lots 1:25,000 2 lots 1:15,000 1 lot 1:10,000 3 lots 1: 5,000* 1 lot 1: 5,000 Average toxicity 1:21,000 Excluding* average toxicity 1:25,000	1 lot 1:25,000 1 lot 1:20,000 1 lot 1:5,000 Average toxicity 1:16,000
Third quarter	2 lots 1:8,000 2 lots 1:5,000 Average toxicity 1:6,500	2 lots 1:60,000 Average toxicity 1:60,000	1 lot 1:20,000 2 lots 1:12,000 Average toxicity 1:14,000
Fourth quarter ...	1 lot 1:60,000 1 lot 1:45.000 1 lot 1:35,000 1 lot 1:25,000 1 lot 1:12.000 1 lot 1: 7,000 Average toxicity 1:30,000	1 lot 1:30,000 1 lot 1:20,000 1 lot 1:15,000 Average toxicity 1:21,000	Production for this quarter not completed

*These three lots were grown at too high temperature, i.e., 42°C.

Berna Pepton

About this time our attention was called by Dr. Noble of the New York State Laboratory to a pepton put on the market by the Swiss Vaccine and Serum Company of Berne, Switzerland. The statement that this pepton was made according to Witte's recipe was received with some skepticism but the thought did occur to us that this might be a means of getting Witte pepton into the Allied countries.

A small amount of broth (about 15 liters), was made up according to our usual method using the "Berna" pepton instead of the Witte. So interested were we in the result, that a small amount was withdrawn from one of the flasks, filtered and tested on the sixth day of growth. The minimum lethal dose was found to be over 1:10.000 after even this short incubation period. At the end of fifteen days the rest of the cultures were filtered and tested. The pig which received one c.c. of a dilution of 1:45,000 died of tetanus on the fourth day. A second preparation of toxin broth made with this pepton yielded a toxin of 1:100,000 in potency.

Broth made with Berna pepton has been used at the Bureau of Laboratories since May, 1918, to the present time with favorable results for the production of tetanus toxin (see table 6). The variations in the quarterly averages were greater than when Witte pepton was employed.

The average toxicities as given in this table showed again that the seasonal factor was of no importance in the production of potent toxins. The second quarter in the years 1918. 1919 and 1920 gave averages of 1:72,000. 1:21,000 and 1:16,000 respectively. The third quarter of these same years showed still greater differences. The average toxicity in this period of 1918 was 1:6,500. In the year 1919 this quarter gave a high average toxicity of 1:60,000 and the same period in the year 1910 showed an average toxicity of 1:14,000.

Summary

In going over the above results, it will be seen that Witte pepton has been an important factor in the production of a fairly constant potent tetanus toxin. The indications are that the Berna pepton is a satisfactory substitute for Witte since the latter is no longer available.

It would seem either that the Swiss Serum and Vaccine Company was justified in its claim that Berna pepton was made according to Witte formula or that Berna pepton was in reality Witte pepton since only when using these two peptons did we obtain a toxicity of 1:100,000.

It was a great disappointment that Martin pepton broth as made by us proved so unreliable. It is just possible that one of the reasons for the better results with this broth at the Pasteur Institute, is due to the fact that a larger number of stomachs are used for the preparation of the pepton than we can handle at one time at the Bureau of Laboratories.

For bibliography see the original article.

TETANUS TOXIN ANTITOXIN MIXTURE FOR ACTIVE IMMUNIZATION IN GUINEA PIGS

By Edwin J. Banzhaf and Chas. K. Greenwald

During October, 1920, tetanus toxin, four months old and testing 4000 M.L.D. per cc. was selected for the preparation of toxin-antitoxin mixtures. These preparations contained respectively 5.5, 6, 6.5 and 6.75 units to each cc. of toxin. Of these mixtures the first three caused acute death in guinea pigs when five cc. was given. The latter two which correspond to about 60 per cent. and 70 per cent. more antitoxin than theoretically required to neutralize all the toxin, caused stiffness in guinea pigs with recovery when five cc. was given.

A mixture was then prepared containing 90% over the theoretical required amount, five cc. of which caused no stiffness in guinea pigs. This over neutralized mixture was used for active immunization in guinea pigs as shown in the following table.

RESULTS OF A SINGLE IMMUNIZING DOSE OF THE OVER NEUTRALIZED MIXTURE OF TETANUS TOXIN-ANTITOXIN

Date Injected October 29, 1920	Dose	9 Weeks Later Injected with 1.5 M.L.D. of Tetanus Toxin	Results
	1/10 cc.	1.5 M.L.D.	Dead 2 days
	1/10 cc.	1.5 M.L.D.	Dead 4 days
	1/10 cc.	1.5 M.L.D.	Dead 6 days
	1/10 cc.	1.5 M.L.D.	Dead 5 days
	1/10 cc.	1.5 M.L.D.	Lived
	1/4 cc.	1.5 M.L.D.	Lived
	1/4 cc.	1.5 M.L.D.	Lived
	1/2 cc.	1.5 M.L.D.	Lived
	1/2 cc.	1.5 M.L.D.	Lived
	5 cc.	1.5 M.L.D.	Lived
		Control 1 M.L.D.	Dead 4 days

Definite evidences of active immunization are shown when only one injection is given and when the mixture is 90% overneutralized. The foregoing preparations were stored in the refrigerator and two years later, on retesting, the mixture containing 5.5 units antitoxin (35% excess) was found to cause slight stiffness in guinea pigs when five cc. was injected.

Guinea pigs receiving a single injection of 1/10 c.c. of above mixture, withstood five M.L.D. of tetanus toxin three months later, without any signs of stiffness.

It is interesting to note that as little as 1/25 of a fatal dose of tetanus toxin in a guinea pig will cause definite stiffness.

MICROBAL STUDIES ON ACUTE RESPIRATORY INFECTION WITH ESPECIAL CONSIDERATION OF IMMUNOLOGICAL TYPES[1]

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William H. Park, Anna W. Williams and Charles Krumwiede

Introduction

Outline of work planned, discussion of results and general conclusion

It was decided at a conference of the members of the Influenza Commission[2] and the Research Laboratory workers that the problems[3] to be investigated in New York City should be the following:

1. A study of the microörganisms of the upper respiratory tract in "health," in "common colds" and in "influenza." This investigation should include the diligent search for an epidemic microbal strain in influenza cases. Both filtrable and unfiltrable microbes were to be considered.
2. A continuation of our studies on hemoglobinophilic bacilli (group of the influenza bacilli) for the purpose of establishing the relationship of the influenza bacillus to both pandemic and sporadic cases of influenza.
3. A study of the permanence of type characteristics of influenza bacilli in persons after recovery from respiratory infections. It was considered of the utmost importance to establish as accurately as possible the stability of types.
4. A study of the incidence of common colds, "influenza," and pneumonia, following the controlled use of a vaccine made up of different types of bacteria claimed to be the cause of acute respiratory diseases.

In carrying out this work, a large part of the material for the first two problems was collected from employees of the Metropolitan Life Insurance Company who volunteered their aid. The rest of the material was collected from laboratory workers and from cases of "influenza" in the hospital for contagious diseases. The preventive value of the vaccine was tested upon 1600 of the employees of the Metropolitan Life Insurance Company.

In arranging the work for the first problem, the investigation of the incidence of microbes was planned to include enough normal cases so as to have for study a sufficient number of persons who would later develop acute "colds" or "influenza" in order to obtain significant comparative curves of microbal incidence beginning in pre-disease stages and continuing throughout the attack; also to note the effect, of any, of vaccination upon the subsequent microbal content of a case. Our plans were changed, however, because of an early outbreak of acute respiratory infections which were diagnosed shortly after as influenza. In our opinion there is considerable doubt as to the influenzal nature of many of these cases. Notwithstanding our large force of workers, we were unable to investigate these and at the same time study normal cases as fully as we had planned. We were afraid to postpone study of the new cases as the outbreak might stop and we should then have had no acute influenza cases for study. We concentrated, therefore, on a mass of "influenza" cases, hoping, in spite of our previous failure, to demonstrate a common microbal strain whether filtrable or non-filtrable.

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[1] A series of papers from the Research Laboratory, Department of Health, New York City, and one from the New York Hospital.

[2] The members of the commission are Drs. M. J. Rosenau, G. W. McCoy, Lee Frankel, A. S. Knight, E. O. Jordan, W. H. Frost and W. H. Park.

[3] The studies are a part of the investigation on the etiology and the prevention of the acute respiratory diseases being carried out in Boston, Chicago, Washington and New York under a grant from the Influenza Commission Fund of the Metropolitan Life Insurance Company.

In the first paper we attempted to show the value of different technical methods in the demonstration of the different species of microörganisms from the upper respiratory tract and to give the average comparative incidence of groups of microörganisms demonstrated by these methods in health and disease. We lay stress on the methods of obtaining and handling the material.

In the succeeding papers in this number of the Journal and in other papers which will appear later, are reported the studies made on the isolations from colonies of the various species appearing on the original plate cultures made directly from the case or from the inoculated mice. The purpose was the search for a common "outbreak" or epidemic strain among the cultures isolated.

In two additional papers in this number are given respectively: A brief history of two cases of accidental infection with the influenza bacillus and the persistence of type characteristics in the inoculated persons; The results of vaccines on the prophylaxis of respiratory inflammations.

Discussion

The chief object of all of these investigations was to demonstrate a common "epidemic" or "outbreak" strain among the groups of microbes claimed to have caused epidemics. As much as we were able, we employed methods that might best demonstrate these groups. We continued to bear in mind, however, that the methods we used might also demonstrate in small numbers hitherto undescribed organisms that might have an etiological relationship to these infections. In spite of this, we decided to study closely only the more dominant types that were demonstrated by our procedure, simply recording the minority groups as determined by colony appearances and the examination of stained specimens from these colonies. We recognized that our methods of collecting material did not take into account the possibility of the primary exciting factor growing chiefly beneath the surface of the mucous membrane. We believe we are justified in assuming the probability that such a specific microbe would predominate or at least be prevalent upon the surface of a localized area of mucous membrane during some part of the early stages of the infection. If this microbe were an aerobe capable of growing on the culture media employed, selected fishings from ten colonies of the dominant types appearing on these media should give a majority of cultures obtained from the epidemic cases having no fundamental differences.

The results of our studies indicate that of the different groups of microörganisms isolated by our procedure, all had the peculiarity that each group was an assemblage of many types. This was equally true for the pneumococcus, the influenza bacillus, the green-producing streptococci and the hemolytic streptococci. We obtained no evidence of the existence of a common filtrable organism. We accepted as the most delicate test of identity of types in strains the presence of specific agglutinable and agglutinogenic substances as determined by direct agglutination and agglutinin absorption methods. Biochemical characteristics are insufficient for such a determination; at most they divide a species only into the broader groups. In our isolation work, we are aware that the number of colony fishings may not have been enough in every group of bacteria to rule out a common strain. In a study of case incidence alone, some groups may be ruled out as implicated in the epidemic; such as staphylococci, Gram-negative cocci and hemolytic streptococci. More work could be done on pneumococci, green streptococci, indifferent streptococci and some of the minority groups with a high case incidence. The question of the relationship between the bacterial types used in vaccination and the microbal strains obtained from the throats of the vaccinated remained mostly unanswered. The specific strains of organism used in the vaccines were not found to any extent in either normals or the diseased. It is a curious fact that in all but one instance the fixed types of pneumococci, as found in eleven of all the cases examined, occurred among the unvaccinated influenza cases. This loses significance, however, in the light of the fact that only 6 of our cultivated influenza cases had received vaccine and 42 had not.

The evidence of immunological response to the vaccine was, as might be expected from the above findings, apparent only in the lessened incidence of pneumonia. The percentage of colds was as great among the vaccinated as among the unvaccinated. The pneumonia incidence was much less. The greater multiplicity of types of microbes believed to be capable of exciting common colds over those usually exciting pneumonia, is possibly the explanation of the apparent uselessness of the vaccines employed in this series in preventing minor respiratory infections while apparently affording considerable protection against pneumonia.

STUDIES ON ACUTE RESPIRATORY INFECTIONS

Methods of Demonstrating Microörganisms, Including "Filtrable Viruses," from Upper Respiratory Tract in "Health," in "Common Colds" and in "Influenza" with the Object of Discovering "Common Strains"

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Anna W. Williams, Mary Nevin and Caroline R. Gurley
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Choice of Methods

From the standpoint of arranging methods, we divided the microörganisms for which claims had been made into two general groups, namely, aerobic and anaerobic microbes, subdividing each into filtrable and non-filtrable microbes. As the most vigorous claims had been made for certain aerobic non-filtrable microbes and for anaerobic filtrable ones, we arranged our methods with special reference to these. We divided the aerobic non-filtrable organisms further into four general groups; namely, Gram-positive cocci, Gram-negative cocci, hemoglobinophilic bacilli and "others." We concluded that one of the nearest approaches to a standard method for demonstrating filtrable organisms from the upper respiratory tract was that reported by Foster (chiefly Noguchi technic). For this reason and because we wished to corroborate his work, we decided to use his method as well as modifications of it.

In regard to demonstrating the aerobic non-filtrable microbes from the mucous membranes of the upper respiratory tract, although no comprehensive method of procedure was found that was described fully enough to be used, we decided that certain culture media with methods of inoculating them might be considered standard. Among these were Brown's technic for the demonstration of Gram-positive streptococci and Avery's medium for hemoglobinophilic bacilli. Other media which were used are described in the original article. The technic for the collection of material and for first cultures is described in the original article.

Results

1. From aerobic cultures of non-filtrable material.

Although the cultural results were influenced greatly by the area from which the material was obtained, the effect of the different kinds of media was equally marked. Thus on our vitamin medium we had abundant growths of all groups, so abundant, that in many cases we had overgrowths. On Avery's oleate agar, as was to be expected with an inhibiting medium, chiefly influenza bacilli and certain Gram-negative cocci and other Gram-negative bacilli grew. It is interesting that most strains of meningococci do not grow well on this medium, while most strains of gonococci and *B. pertussis* do. On veal blood-drop plates, by our procedure, the proportion and size of influenza bacilli colonies were less, while in Brown blood pour plates the influenza group and all other groups, except the Gram-positive cocci, appeared usually in still smaller numbers. This was due, partly, to our manner of inoculating the material but in a series of cases where we put the swab from the case directly into the diluent (pneumococcus broth) and inoculated equal proportions of broth in each medium, our results were practically the same. The copy of one of our primary case records indicates these differences.

2. From anaerobic cultures of non-filtrable washings.

No filtrable organisms were found.

3. From anaerobic cultures of filtered washings.

In six out of the forty cases cultured in this manner, anaerobic organisms were isolated. Two cases yielded a Gram-positive non-hemolytic streptococcus, one other case a diphtheroid; two cases a Gram-negative coccus and one a case a small Gram-positive coccus. In only one instance was the growth found in more than one of the inoculated tubes and in this instance only two showed evidence of growth. The controls remained sterile. Meat medium cultures of all of these organisms were filtered through Mandler filters. No growth occured in any of the cultured filtrates.

No further work was done with these cultures because they seemed to be of no etiologic significance. On the contrary, they were regarded as contaminants, although all of the control tubes (2 meat and 3 horse serum glucose agar) in each case remained sterile.

4. From anaerobic cultures of filtered washings. (Noguchi techinc chiefly).

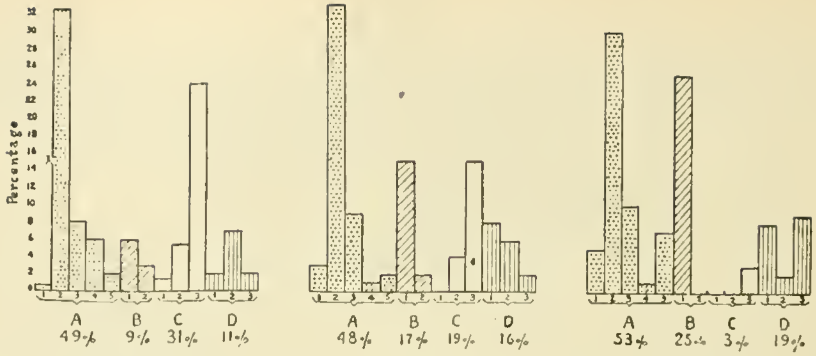
Growths were obtained in the anaerobic cultures of filtrates from 4 out of 10 consecutive cases of influenza. In all of these cultures, a minute coccoid organism, differing somewhat morphologically in two of the cases, was found. These were all obtained from the filtrate of the nasopharyngeal washings but they could not be carried on for more than one culture generation. No animal inoculations were attempted.

Of 23 early rhinitis cases, one showed an anaerobic coccus which died out in the third culture generation; one other case, a coccus, probably anaerobic, which died out in the second culture generation. In 20 normal washings no organisms were found.

From the filtrates of 7 early "cold" cases of a type similar to that described by Foster and from three typical influenza cases, we were unable to infect 45 human volunteers, 20 of whom were students less than 30 years of age. In the method of collecting the material and doing the inoculations Foster's technic was followed as closely as possible.

Comparative Incidence of Microorganisms from Nasopharynx as Recorded in Our Original Records

In trying to indicate the changes which we found in "normals," "colds" and "influenza" in the incidence of the group of microörganisms according to the chart headings, we obtained from our primary cultures the common incidence in each group by estimating the average percentages for each case on all the culture media used, adding these percentages in each individual group and dividing the sum by the total number of cases in that group. The following chart gives this common incidence in percentage for each group of cases.



"Normals" "Colds" "Flu"

Chart Showing Comparative Incidence of Microörganisms by Groups (Reprinted by courtesy of Journal of Immunology)

EXPLANATION OF CHART

A = *Gram-positive cocci.*
    1. "Hemolytic streptococci" (Smith and Brown's beta type).
    2. "Green producing streptococci" (Smith and Brown's alpha type).
    3. Pneumococcus group.
    4. Staphylococcus group.
    5. "Indifferent streptococci" (Brown's gamma type).

B = *Hemoglobinophilic bacilli.*
    1. "Typical."
    2. "Atypical."

C = *Gram-negative cocci.*
    1. Meningococcus group.
    2. Micrococcus catarrhalis group.
    3. *M. flavus, M. siccus* and others.

D = *Other microorganisms.*
    1. "Large Gram-negative bacilli."
    2. *Bacillus mucosus* group.
    3. "Others."

INTERPRETATION OF CHART

In the first place it should be remembered that the chart and the table give no indication of the amount of growth. That was recorded on the original sheets and may be summed up as follows: From the nasopharynx of the "normal cases," on the whole a very scant growth was obtained. In the dilutions we employed, we often obtained no growths on our Brown plates, in fact, from some cases there was practically no growth on any plates, except on the vitamin-blood-agar and the oleate, where the growth was a scanty one. In the "colds," the growth on the whole was very abundant, though in some of the cases obtained in the first hours of the disease, the amount was scanty. In all of the influenza cases, the growth from the nasopharynx was very abundant.

In studying the comparative incidence in the types of clinical cases of the four groups of microörganisms as shown by the chart, we note that there are few differences if the groups are considered as a whole. We see simply that the Gram-negative cocci group drops somewhat in the "cold" cases and markedly in the influenza cases, while the influenza bacilli and the "others" progressively increase. When we study the subgroups, however, more differences appear. But we must take into account more minute colony characteristics in order to appreciate further differences.

Thus, among the Gram-positive cocci, the "alpha type" of streptococcus (green-producing streptococci) predominate in all three kinds of cases. But in the "normals" and "colds," this type varied in general colony characteristics in the different cases while in practically every influenza case the majority of green-producing colonies seemed to be of one type and of a type that had not appeared before, at least in any numbers. We labelled these colonies "minute green" and these are the colonies from which isolations were made for further study and comparison with the fishings of green-producing colonies which we had made from the "normals" and "colds." (See paper III of this series.)

The "beta type" of streptococcus shows a small increase when compared with group incidence, although in disease the increase over its appearance in normals is marked. It is significant that out of 12 cases of colds in which hemolytic streptococci appeared in the cultures from the nasopharynx, 7 of the cases began as tonsillitis. The further study of the beta streptococci from this series of cases is to be reported later.

The pneumococci show a very slight increase in "colds" over the "normals" and in "flu" over the "colds." The further study of the strains is given in Paper II of this series. No common type was found. It is interesting that of the few fixed types found in the influenza cases, all occurred in the ten cases not inoculated with our vaccine (see Povitsky paper), but, as only 6 of the 48 cases in this series had been inoculated with the vaccine, this observation has little, if any, significance.

Since the percentage of staphylococci were infrequently demonstrated from the nasopharynx in disease, we decided to discontinue the study of the individual fishings.

Although there was a marked increase of the "indifferent streptococci" (Smith and Brown's gamma type) in "colds" and "influenza," over the percentage usually found in "normals," there were very few in the individual cases. However the increase occurring in "flu" case incidence indicates that a minority curve might be found that may be of significance.

That among these colonies diagnosed originally as "indifferent," that is, producing no changes on standard blood-agar-plates, some may be found on further study to be alpha streptococci type, is possible. We fished a number of these colonies from many cases and studied primary cultures in a general way hoping to be able to classify them further, but we had to discontinue their study because of the emphasis we were placing on majority incidence.

In regard to the hemoglobinophilic bacilli the most interesting point brought out in the chart, is that the atypical influenza bacilli (see Povitzky's paper) occur less commonly in the "cold" cases than in the "normals" and drop out entirely in the primary cultures from the influenza cases which were studied. This makes the increase in the typical influenza bacilli in the "influenza" cases very marked. The case incidence is 92 per cent. Of course, we show (Povitzky's paper) that there is no one serologic type in this series of cases, thus corroborating our earlier work.

In some of the early "cold" cases, we found large numbers of *M. catarrhalis*. Chiefly for this reason and partly because claims had been made for Gram-negative cocci as an etiological factor, we started to study individual fishings of this group. We studied them for only a short time, however, owing to the fact that neither they nor practically any kind of Gram-negative coccus appeared in our "influenza" cases.

Although types of Gram-negative cocci may have a relationship to certain cases of "common colds," judging from the comparative incidence none had anything to do with the influenza cases in this series.

Among the "others" we recorded and fished colonies of large Gram-negative bacilli showing similar characteristics. These bacilli occurred in increased abundance over normals in "colds" and "influenza" cases with a corresponding increase in case incidence. We made many fishings from this type of bacillus but were unable to finish our studies on it. We have concluded that it may be an important factor in "common colds."

B. mucosus, we recorded because of its relatively abundant growths in certain cases in the "normals" and "colds" but we made no study of individual fishings. It dropped almost out of sight in the influenza cases. The last column in the Chart in the groups of "normals" and "colds" is a record of different organisms from individual cases, some Gram-negative bacilli, some Gram-positive bacilli which were different in the different cases. On the other hand, in the "influenza" cases, this record is due chiefly to "minute" delicate colonies appearing on our first vitamin-blood-agar plates in close proximity to colonies belonging to other groups. When we diluted the material further they dropped out. We attempted to isolate these organisms and obtained in the majority of cases a minute cocco-bacillary form of short chains and clumps. These died out quickly (we made the majority of our fishings on chocolate agar) and as they were in such great minority and we were studying majorities we discontinued their study.

CONCLUSIONS

The procedure for collecting and handling material as outlined in this paper is a more comprehensive one than has been given before for demonstrating the comparative incidence of the great groups of microörganisms that have usually been found in the majority of acute upper respiratory infections. Therefore, the colony fishings are more likely to be representative of a dominant type, if one exists.

The procedure has also demonstrated increased incidence in mucous membrane inflammations of certain minority groups due to an increase of one type of organism which might have a relationship to acute inflammations of the upper respiratory tract.

The negative results obtained by our procedure for demonstrating filtrable microbes, we consider an indication that the positive results already reported do not bear as wide an application as their sponsors infer.

For bibliography see the original article.

A STUDY OF THE SEROLOGICAL RELATIONSHIPS OF PNEUMOCOCCI FROM THE UPPER RESPIRATORY TRACT WITH SPECIAL REFERENCE TO COMMON COLDS AND INFLUENZAL CONDITIONS

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This study forms a part of a general investigation by the Bureau of Laboratories and the Influenza Commission of the Metropolitan Life Insurance Company of the flora of the respiratory tract in normal and pathological conditions. The purpose of this section of the work was to determine whether any one variety of pneumococcus would be encountered more frequently in epidemic inflammatory conditions resembling influenza than in those of the nature of ordinary colds and in the normal controls. If, in a wave of common colds or influnezal-like inflammations, a dominant variety could be demonstrated in the majority of cases, it would suggest strongly that the pneumococcus strain in question was the primary causative agent of the outbreak in which it was prevalent. The presence of a single type of a pathogenic variety in the individual case may indicate, however, that it is either the primary agent or one of the secondary invading bacteria, or it may be only an accidental parasitic variety. The problem was, therefore, to determine the similarities or dissimilarities among the various strains isolated from the nasopharynx of normal individuals and of cases of rhinitis, pharyngitis, influenza and broncho-pneumonia. In all instances the nasopharyngeal material examined was the same as that from which the other species of organisms were isolated by the different workers engaged in this general investigation. The methods employed for the study of the similarities and dissimilarities of the pneumococcus strains were those of agglutination and agglutinin absorption.

The study of the strains was based on the facts already known concerning the differentiation of the pneumococci. These facts may be briefly reviewed as follows. Park and Williams classified *Streptococcus mucosus* as a definite variety of pneumococcus, which variety is now known as *Pneumococcus mucosus* or Pneumococcus, type III. Collins, resorting to agglutination and agglutinin absorption with monovalent rabbit serums, found specific relationships among members of this mucosus group and group relationships with other varieties of pneumococci. Cole and Hanes also demonstrated marked cross agglutination among members of this group. Neufeld and Händel also found specific types of pneumococci, which types were influenced only by their own antiserums. These observations were confirmed and extended by Cole and by Dochez and Gillespie who showed that many pneumococci could be placed in two distinct serological types which they termed type I and type II. They defined type 3 as a distinct morphological variety and placed the strains which could not be classified with the above types in a heterologous group of strains termed group IV.

Pneumococci giving a partial or delayed agglutination with type II serums were later defined by Avery as atypical type II or subgroup II strains. According to him, type II serum protected mice against lethal doses of the majority of these strains. He classified ten strains in two distinct groups, subgroup A and subgroup B and a third heterologous group subgroup X. Subgroup A removed from type II serum the agglutinins for other members of that group but not for subgroup B. The subgroup B acted vice versa. However, neither of these removed the specific agglutinins for type II strains. With monovalent rabbit serums for the members of the subgroups, he found that subgroup A strains gave marked agglutination with antiserums for members of that subgroup; that subgroup B strains agglutinated with subgroup B antiserums but there was no cross reaction between these subgroups. Typical type II strains gave no reaction with the antiserum of either subgroup. Stillman following the methods of Avery classified 204 strains in twelve subgroups, the subgroups II-A and II-B of Avery and ten others, subgroups II-C, D, E, F, G, H. J, K, L, and M. The attempt to demonstrate definite subgroups among the strains provisionally classed as group IV, is considered later.

The literature on the incidence of the types of pneumococci in pneumonia is very extensive. The incidence of the different types in normal individuals, including healthy contact carriers, has also been studied in detail but there is little information available on the occurrence of types in other conditions. Park and Williams in 1905 investigated the pneumococci isolated from inflammations of the upper respiratory tract. At that time the separation of types had not been completed and correlation of their groups with those now recognized is not possible. Valentine investigated the incidence of types of pneumococci in common colds with a view to their being a possible source of contagion for lobar pneumonia. She found pneumococci in forty-three out of sixty-five cases of common colds. The incidence of types was as follows: Group I, two cases; group II, two cases; group III, four cases; and group IV, thirty-five cases. Clough published a study in which a few conditions other than pneumonias were included, but for the most part the conditions investigated by her do not coincide with those studied by us. In five cases of chronic bronchitis she found three type IV strains and two strains classed as miscellaneous. In two acute bronchitis cases, one strain was an atypical type II and the other a type IV.

Of 138 cases cultured by us, pneumococci were found by the methods employed, to be present in fifty-three. The criteria adopted by us for identification of an organism as a pneumococcus were typical morphology and bile solubility. All of the cultures fermented inulin, but a strain would not have been discarded had the ability to ferment this carbohydrate been lacking. In one instance a culture was lost before typing was completed; in five the pneumococci were not isolated in pure culture because the plates were overgrown by *B. subtilis* or *B. proteus*. In these instances, it was inferred that pneumococci were present from the discovery of typical Gram-positive encapsulated diplococci in the smears and from the bile solubility of the cocci obtained from the peritoneal washings of the injected mice.

Strains were obtained, therefore, from forty-seven cases. These strains were then tested with serums of the three types. For the identification of the fixed types, undiluted antipneumococcus horse sera were employed and the tests were incubated at 45°C. Of the forty-seven strains, nine were found to belong to the fixed types as shown by the prompt reaction with one or the other serum. These nine were distributed as follows: Type 1 pneumococci were isolated from two normal cases and one influenza; type 2 from two influenza cases; type 3 from four cases—one chronic rhinitis, one acute rhinitis, one pharyngitis and one influenza complicated by bronchopneumonia. From the last case, a pneumococcus of subgroup IIB was isolated as well as pneumococcus type III. These results are included in the summaries (tables 1 and 2) and are discussed later in connection with the findings from further study of the other strains which did not fall into the fixed types.

The thirty-eight remaining cultures were further tested with type serums. The technic employed is given in the original article.

Every effort was made to obtain the highest possible titer. In some instances, a titer of 1:200 was the highest obtainable. Serums less potent than this were not used.

The result of the agglutination tests with these serums are given in a table in the original article.

In the instances where cross agglutination was marked, the specificity of the reactions was investigated by agglutinin absorption tests. The technic is given in the original article.

To summarize: Among the strains not belonging to the fixed types but which were covered by the monovalent serums available, were found six small groups and thirteen strains unrelated one to the other or to the groups just mentioned. This gives a total of nineteen types. Of these, one group and two separate strains belonged to the subgroup II classification. Five groups and eleven unrelated strains were provisionally placed in group IV. Of these, two groups and four unrelated strains belonged among those which gave slight agglutination with type II serums but were not further investigated as to whether they were true subgroups of type II. The strains of another group and one unrelated strain gave slight agglutination with all three type serums. Finally two groups and six unrelated strains comprised those which did not agglutinate at all with the fixed type serums.

There were, in addition, nine strains which were not similar to any of the strains for which there were antiserums. As no antiserums were prepared for these nine, the relationships among them could not be investigated. It is reasonable to suppose that further tests with these strains would yield results similar to those found among the strains for which antiserums were produced.

The results from this part of the investigation are collected in table 1 and are summarized with reference to type and pathological condition in table 2. From these data, the prevalence of a particular type of pneumococcus in any of the conditions studied is not indicated.

In addition to the classification of strains from different cases, a brief study was made of strains isolated from the same case to determine whether several varieties of pneumococci would be found in the naso-pharynx. Colonies morphologically different as far as possible were chosen, varieties of contour, size and color being noted. The agglutination of the strains from individual cases with the antiserum homologous to one strain from each case are given in table 3. In other cases, a serum was considered homologous even if not actually prepared with a strain from a case, if the absorption of the serum by a strain of the case in question was complete. The results with serums considered homologous on the basis of absorption are given in table 4. In but one case was there encountered more than one variety of pneumococcus. In all cases where a subsequent examination of an individual was made, the same variety was isolated again. The instance in which two varieties were encountered has been noted.

For the most part, the fishings considered in tables 3 and 4 were made from a single collection of material. In a few cases, material for cultures was

TABLE 1

TABULAR SUMMARY—VARIETY OF PNEUMOCOCCUS FROM CASES IN RELATION TO CONDITION

NORMAL		CHRONIC RHINITIS		ACUTE RHINITIS		PHARYNGITIS	
Case number	Type	Case number	Type	Case number	Type	Case number	Type
36	Type I	83	Type III	49	Type III	125	Type III
41	Type I	23	Group IV (5)	17	Group IV^{11} (3)	107	Subgroup II B (1)
57	Group IV^{11} (het)	62	Group IV (6)	82	Group IV^{11} (het)	131	Group IV (?)
72	Group IV^{11} (het)			89	Group IV^{11} (het)		
34	Group IV* (?)			94	Group IV^{11} (het)		
69	Group IV* (?)			39	Group IV* (?)		
76	Group IV* (4)			65	Group IV* (?)		
2	Group IV (het)			78	Group IV* (?)		
71	Group IV (het)			99	Group IV* (?)		
				38	Group IV (5)		
				130	Group IV (6)		

RHINITIS AND PHARYNGITIS		INFLUENZA		INFLUENZA AND PNEUMONIA		PNEUMONIA	
Case number	Type	Case number	Type	Case number	Type	Case number	Type
18	Group IV* (?)	164	Type I	157	Type III and	86	Group IV (het)
169	Group IV (6)	144	Type II		Subgroup II B (1)		
		146	Type II	168	Subgroup II A		
		90	Subrgoup II H	161	Gruop IV^{11} (3)		
		135	Subgroup II B (1)				
		166	Subgroup II B (1)				
		134	Group IV^{11} (2)				
		158	Group IV^{11} (2)				
		154	Group IV* (4)				
		159	Group IV* (?)				
		165	Group IV* (het)				
		166	Group IV (het)				
		160	Group IV (het)				
		163	Group IV (het)				

IV^{11} Strains which gave some agglutination with type II serum.

IV* Strains which gave some agglutination with all three type serums.

Figure in parentheses is an arbitrary symbol to note the groups encountered, based on agglutinin absorption.

(Het) Heteroganous on serum reactions.

(?) No homologous serums available, did not show relationships.

taken a second or third time after intervals as recorded in the tables. Attention must be called to the fact that in many instances the strains of pneumococci were obtained from mice injected with the original material. That by this method only one variety was recovered from a case was not surprising. It was to be expected that the mouse would serve in a selective manner; the most virulent variety would most likely be recovered in predominance or alone. However, where pneumococci were obtained from plates inoculated directly with the material from the nasopharynx, the results were the same as after mouse passage.

The results of previous workers as regards the fermentative reactions of pneumococci do not suggest that these reactions would be of value in the differentiation. Park and Williams found some differences in mannite fermentation; about 70 per cent of their strains fermented this alcohol. All their mucosus types gave a positive reaction. Avery[3] found differences in fermentation with mannite and salicin but these differences did not correlate with the serological results.

In spite of the above data, it seemed desirable to determine the fermentative characteristics of the strains classified by us as distinct groups on the basis of agglutinin absorption. Similar groups have not been studied in this connection; the greater attention has been placed on the dominant fixed types. From one to six strains of an individual case were employed.

TABLE 2

TABULAR SUMMARY: NUMBER OF STRAINS OF EACH VARIETY IN RELATION TO THE CASE CONDITION

GROUPS	NORMAL	CHRONIC RHINITIS	ACUTE RHINITIS	PHARYNGITIS	RHINITIS AND PHARYNGITIS	INFLUENZA	INFLUENZA AND PNEUMONIA	BRONCHO-PNEUMONIA	TOTALS
Type I	2	—	—	—	—	1	—	—	3
Type II	—	—	—	—	—	2	—	—	2
Type III	—	1	1	1	—	—	1×	—	4
Subgr. II A	—	—	—	—	—	—	1	—	1
Subgr. II (B) (I)	—	—	—	1	—	2	1×	—	4
Subgr. II H	—	—	—	—	—	1	—	—	1
Group IV^{11} (2)	—	—	—	—	—	2	—	—	2
Group IV^{11} (3)	—	—	1	—	—	—	1	—	2
Group IV^{11} (het.)	2	—	3	—	—	—	—	—	5
Group IV* (4)	1	—	—	—	—	1	—	—	2
Group IV* (het.)	—	—	—	—	—	1	—	—	1
Group IV* (?)	2	—	4	—	1	1	—	—	8
Group IV (5)	—	1	1	—	—	—	—	—	2
Group IV (6)	—	1	1	—	1	—	—	—	3
Group IV (het.)	2	—	—	—	—	3	—	1	6
Group IV (?)	—	—	—	1	—	—	—	—	1
Total	9	3	11	3	2	14	3	1	

See Table I for explanation of symbols.
× Types isolated from the same case.

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[3] Results not published. Personal communication.

TABLE 3

AGGLUTINATION OF STRAINS FROM INDIVIDUAL CASE WITH ANTISERUM FOR ONE STRAIN FROM THE SAME CASE

CASE NUMBER	EXAMINATION NUMBER	DAYS ILL	ISOLATED FROM	SERUM USED	AGGLUTINATION TITRE OBTAINED WITH STRAINS NUMBERED							
					1	2	3	4	5	6	7	8
168	II	15(?)	m	168-V-1		500	500	500				
168	IV	22(?)	m	168-V-1	600	500		600				
166	I	3	m	166-II-I	200	100	200					
166	II	7	m	166-II-I	200	100						
166	III	13	m	166-II-I	200							
90	III	4	m	90-III-1	500	500	500	500				
158	I	2	m	158-I-I	400	300	400	400				
158	II	7	m	158-I-I	400	400	400	300				
17	I	1	m	17-I-1	400							
17	I	1	pl	17-I-I		400						
72	II	—*	m	72-II-4	500	600	600	600				
89	II	2	m	89-II-I	300							
89	III	?	m	89-II-1	300	300						
57	I	—	m	57-I-5	200	200	200	200	200	200		
94	I	1	m	94-I-5	300	300	300	300	300	300		
76	I	—	m	76-I-1	300	300	300	300	300	300		
154	I	1	m	154-I-1	400	400	400	400	400			
165	I	4	m	165-I-1	200		200					
38	II	½	m	32-II-2		400	400	400	400			
52	I	?	m	52-I-1	200	200	300	200	200			
130	I	¾	m	130-I-1	600	600	500	600	700			
2	II	—	m	2-II-1	400	500	300	400	500			
71	I	—	pl	71-I-5	400		400		400	400		
86	I	9	pl	86-I-1	300	400	300	300	300	400	300	
156	I	2	m	156-I-1	300	200	300	300		300		
160	I	2	pl (a)	160-I-1 (a)	300	200	200	200	200	200	300	200
160	I	2	pl (b)	160-I-1 (a)	200	200	200	200	200	200	200	260
160	I	2	m	160-I-1 (a)	200	200	300	300				
163	I	2	m	163-I-1	200	200	200	200				

*Symbol — = normals.

TABLE 4

AGGLUTINATION OF STRAINS FROM INDIVIDUAL CASE WITH ANTISERUM FOR STRAINS SHOWN TO BE THE SAME ON AGGLUTININ ABSORPTION

CASE NUMBER	EXAMINATION NUMBER	DAYS ILL	ISOLATED FROM	SERUM USED	AGGLUTINATION TITRE OBTAINED WITH STRAINS NUMBERED				AGGLUTINATION TITRE, SERUM STRAIN
					1	2	3	4	
135	I	1/24	m	166–II–1	300	300	200	200	300
157	II	8	m	166–II–1	300	300	300	300	300
134	I	1/6	m	158–I–I	300	300	330	300	300
161	I	1	m	17–I–I	300	400	400		300
161	III	7	m	17–I–I	300	400			300

All the strains except two, promptly and vigorously, fermented inulin, lactose, raffinose and saccharose. The two strains from case 169 showed less avidity for inulin than the other strains. These positive results are not tabulated. The strains showed considerable variation however in their ability to ferment salicin and mannite. These results are given in the original article.

The results show a complete lack of any correlation between fermentative reaction and agglutinative results. There is a marked tendency for the results to vary on different media as well as upon the same medium. A few of the strains show a greater and a more constant tendency to ferment. Variable results were obtained even with individual fishings from a single case.

On the whole, therefore, the conclusion seems justified that the ability to ferment salicin is not a fully developed characteristic. The ability to ferment mannite is even less developed. The condition is comparable to the variable avidity for certain carbohydrates or alcohols shown by members of the typhoid-paratyphoid dysentery groups.

Conclusions

The serological relationship of fifty-five strains was studied. These were isolated from the naso-pharynx of normal controls, from cases of rhinitis, and pharyngitis, also from influenza-like cases and influenza-like cases complicated by broncho-pneumonia. No single variety of pneumococcus was found to be dominant in the inflammatory conditions studied. This was as true for the influenza-like cases as for common colds. At most the only difference between the normal and the acute inflammatory condition was a slightly greater incidence in the latter of the different varieties classified as sub-groups of type II. There were no data, therefore, which indicate that the pneumococcus was the primary etiological agent in the contagious types of inflammation.

In twenty-five cases from which several strains were isolated from each individual, all the strains from the individual case were similar with one exception. In this case two varieties were found. These findings indicate that usually one variety of pneumococcus predominates in the naso-pharynx.

Some of the serological groups as well as individual strains, have been classified as members of the sub-groups of type II by the use of control strains. Other groups and individual strains seemingly should be classified in the same manner. Some groups belong apparently in group IV. The question whether strains should or should not be classified as belonging to the sub-groups of type II is not satisfactorily determinable with the criteria available.

The fermentative results with salicin and mannite were inconstant and the differences among strains showed no parallelism with the serological findings. Such differences have, therefore, no evident classificatory significance.

For bibliography see the original article.

RELATIONSHIP TO UPPER RESPIRATORY INFECTIONS OF STREPTOCOCCI PRODUCING A GREEN ZONE ON STANDARD BLOOD AGAR PLATES (SMITH AND BROWN'S ALPHA TYPE)

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The primary object of this study, as we have already stated in the introduction, was to look for evidence of an epidemic strain among cocci of this type.

With regard to this group of bacteria, perhaps more than any other, sweeping statements have been made concerning the relationship of strains to epidemics. This is largely due to the ubiquity and the difficulty of demonstrating clear cut proofs of such relationship and also to the ease with which deductions are made from insufficient evidence.

The most important question relating to this subject about which we have little accurate knowledge, is the question of the permanency of those characteristics which are supposed to be indicative of species, of specific pathogenicity and of related characteristics which might be used in diagnosis.

We know a little of the limits of change in *vitro* but essential changes in the animal have not yet been demonstrated with controls sufficiently free from error. Indeed, until we can employ a technic that seems now to be practically impossible in its minutia, we cannot claim to have demonstrated fundamental changes occurring in microorganisms grown in either test tube or animal.

So much has been claimed for this group of organisms, especially since the "flu" pandemic, that it seems more important than ever to obtain a more definite knowledge of them. The accounts of the strains reported as probably having an etiologic relationship to influenza are so meager that we cannot agree with a recent statement that these strains are "probably all the same organism," especially in the light of our work on colony fishings, the first report of which appears in this paper.

The most minute recent work on cultural characteristics of "green producing streptococci" has been done by Brown, confirming and continuing the work started by Theobald Smith and Brown in 1915. The chief advance that Brown made (1919) in studying more minutely the changes on a standard blood agar plate was to describe certain definite ice-box changes in the colonies. In his very comprehensive monograph he gave an extensive comparative table of bibliography and tried to correlate his work with that of others. He adopts, with some modifications, the classification of Holman as to the sugars.

His work on colony changes on his standard blood agar plate is most interesting in that he claims that certain small but definite appearances differentiate clearly between the so-called true hemolyzers (Smith and Brown's beta type) and the green-producing streptococci (Smith and Brown's alpha type).

We are using the term "alpha" as stated by Smith and Brown in 1915, although Brown in the introduction of his 1919 monograph says that they avoided the term *Streptococcus mitior seu viridans* for the less hemolytic streptococci because many of them produced little or no green color in blood agar plates. All of our strains reported in this paper were fished as green-producing colonies from Brown's standard-blood-agar plates. We are also accepting tentatively the broader description of hemolysis that Smith and Brown recognize, namely, that of a passing of hemoglobin from the red blood cells from whatever cause and that any lightening of the medium about a colony in a standard blood agar plate is evidence of a corresponding degree of hemolysis in this general sense.

As we said, the strains reported in this paper were originally fished from colonies that appeared to be of the alpha type and in the influenza cases all apparently of one type of alpha which we labeled "minute greens." These fishings were subsequently studied with great detail on blood agar plates following exactly Brown's method as reported in his monograph.

They were also studied for morphologic and staining characteristics after growth on chocolate agar and in pneumococcus broth. They were all Gram-positive cocci growing chiefly in medium-length chains in the broth and in pairs on chocolate agar. On the latter medium the individual organisms were frequently elongated. The morphology of the organisms in a few of the isolations differed in some particulars from that of others but whether these are lasting differences we do not know. The bile test in all of them was negative. Their reactions were tested to seven carbohydrates, namely, dextrose, saccharose, mannite, lactose, salicin, raffinose and inulin. Duplicate tests were made with the addition of serums from defibrinated horse blood.

Smith and Brown had described a sub-group of their alpha type based on colony formation which they called "complex." Brown described a sub-group based on blood change which he calls "alpha prime."

In general, we may say, our description of the types agrees with that of Brown. The slight differences we have found may be listed as follows:

1. All the strains we have studied as "alpha" produce a green zone, however delicate, on standard plates.
2. Some of our alpha strains have shown no increase in the zone of clearing after refrigeration.
3. Our alpha strains growing as "complex colonies" are rather frequent.
4. All of the strains, which we have classed as "alpha prime," have produced at least some green color within forty-eight hours in the incubator (36°C.).
5. Some of our alpha primes have produced some hemolysis in the test tube (reported to us by Mishulow).
6. An occasional definite "beta" strain has produced a green zone on the standard plate.
7. Two strains of "betas" have markedly complex colonies. (These are the strains marked "Witt"[3] and "Witt-like" in the report to be given by Valentine and Mishulow.)

We have come to the conclusion that the chief distinguishing characteristic between the alpha and beta type of hemolysis on Brown's standard blood agar plate is the zone of corpuscles immediately surrounding the colony, and that this characteristic may be a more constant test of the different types than tube hemolysis.

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[3] Two interesting organisms were obtained from the "Witt" case, with which different groups in our laboratory have worked. One is a hemoglobophilic bacillus (see Dr. Povitzky's paper) and the other is a hemolytic streptococcus.

We found that there was no consistent relation between types on standard blood agar plates and sugar reactions. Heterogeneity of sugar types found in colony fishings of "green streptococci" from individual cases of "common colds" has been reported by Krumwiede and Valentine.

The only general statement we can make so far from this study is that in a series of influenza cases, cultures isolated from colonies of an apparently similar type belonging to the "alpha streptococci and appearing as dominants, show a multiplicity of strains as indicated by the reactions with carbohydrates and with a standard blood-agar medium.

Although these cultural reactions may not be as fixed as certain immunological reactions, it remains to be demonstrated that these cultural reactions can change in their passage from individual to individual throughout a single epidemic.

For bibliography see the original article.

ON THE EXISTENCE OF A MULTIPLICITY OF RACES OF B. INFLUENZAE AS DETERMINED BY AGGLUTINATION AND AGGLUTININ ABSORPTION.

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In the general investigation of the bacteriology of the recent epidemic of influenza, carried out under the supervision of Dr. Wm. H. Park and Dr. Anna W. Williams, it was early seen that one of the most important questions was the identity or non-identity of the strains of *B. influenzae* from the epidemic cases. Although the practically uniform presence of *B. influenzae* in actual cases of the disease suggested the etiological importance of this organism, no conclusion was possible without the further demonstration that the strains encountered were identical. It would be difficult to conceive a pandemic spreading from country to country, due to different races of a microörganism.

Preliminary observations indicated that the relationship of the strains isolated could be determined most easily by agglutination and agglutinin absorption. It was found that agglutinating sera, active in dilutions of 1—800 to 1—1000, could be produced without great difficulty by intravenous injection of rabbits, starting with live or killed bacilli. The technic employed is given in the original article.

The first strains selected for study, with one exception, were isolated at post mortem from lung or larynx. If there were an epidemic strain it seems that the strains having the ability to extend from the nasopharynx and invade the lung would most likely be the actual epidemic variety rather than an accidentally present parasitic type. As has been shown, the influenza bacillus is very frequently encountered in the nasopharynx of apparently normal individuals as well as in the throats of persons suffering from the diseases due to other agents. The preliminary strains were nine in number, and an agglutinating serum was prepared for each. For comparison, a culture of *B. influenzae* (B. I.) isolated in 1914 from the lung at autopsy in a case of pneumonia and a serum prepared against the culture were included.

The general absence of cross agglutination, or the slight degree when present, as shown in this series, indicates that even among these ten strains there exist dissimilar varieties.

To exclude the possibility that the cross agglutination obtained was any indication of close relationship or identity, agglutinin absorption was resorted to.

The results indicated that the ten strains studied were different one from the other, or in other words from nine cases of influenza, nine distinct races of the influenza bacilli were isolated. This result was so surprising that it seemed advisable to extend the study to another series of cultures, especially those isolated in the early stages of the disease, and to strains isolated from cases during convalescence, as well as those isolated from apparently normal throats. An additional series of cultures obtained post mortem from lung, larynx, or trachea, together with six strains isolated by means of mouse passage from sputa of suspected influenza pneumonia were included.

Two antisera prepared with nasopharyngeal strains isolated early in the disease were employed in addition to the nine sera employed in the previous series.

The results again indicated relatively little relationship between the strains employed and the strains used for the antiserum. In some instances, a considerable degree of cross agglutination was noted. To determine whether this indicated a close relationship or identity, absorption was resorted to.

In only two instances, was identity in absorptive capacity encountered.

Although these two instances of identity were encountered, the general absence of relationship of the strains from this series of cases to the strains used to produce the antisera was strongly suggestive of multiplicity. A possible explanation would be that not one of the serum strains used was an actual primary infecting epidemic strain but rather a secondary infecting strain of the same species. If *B. influenzae* were the actual primary etiological agent of the disease, the explanation just referred to would seem somewhat remote, in view of the origin of most of the strains from post-mortem cases which is evidence that the strains possess invasive power. To obtain more direct evidence as to the multiplicity of the strains of *B. influenzae* as well as on the question of the possible occurrence of primary and secondary infecting strains of this microörganism, two further series of cases were investigated.

Two series were studied, one from a group of marines who had lived for some time in an isolated camp, the other from the inmates of an infant asylum. Two or more isolations were made from a considerable number of the cases. If a definite strain was the primary etiological factor, it would not seem possible to fail to demonstrate a considerable degree of identity in the strains, in such series. Antisera were prepared with four cultures from the marine group and with one culture from the asylum group.

The cross reactions were no more marked than in the previous series with the exception of one antiserum of the marine group. This serum gave many marked cross reactions, several equalling or exceeding the reactions obtained with the homologous strain.

Agglutinin absorptions were again carried out in the instances where possible relationships were indicated. In the marine group, two strains from different individuals were found to have the same agglutinin absorptive capacity (M. 1 and M. 4). Two strains from different individuals were also found to be identical in the asylum series.

In spite of the marked cross agglutination shown by one antiserum, none of the strains from other individuals absorbed the specific agglutinins of this serum. In other words in spite of close contact no greater proportion of identical strains was encountered in this series than in the general series already described. A noteworthy finding is the result obtained with three successively isolated strains from a marine. Two earlier isolations were alike, whereas the later isolation was a different type. The appearance of different strains in the throat is not necessarily an indication of primary and secondary infection by two strains of the same bacterium. The individual may have possessed the two strains originally, or the second strain may have been implanted by contact.

An opportunity arose to study the strains isolated from the members of a single family all ill with influenza. The onset of the disease in the different members was very close together.

Antisera were prepared with the strain isolated from each individual. No identities were encountered. It would seem that in such a series infected from a common source or one from the other, identities should be encountered, if B. *influenzae* were the primary infectious agent.

TABLE 1

Summary of Investigation

SERIES	NUMBER OF STRAINS TESTED	NUMBER OF ANTISERA FOR GROUP	NUMBER OF OTHER ANTISERA	FINDINGS
Autopsy	10	10	7	All strains found to be distinct races. One autopsy strain identical with a miscellaneous strain. Another autopsy strain identical with another miscellaneous strain.
Miscellaneous	73	0	18	No two strains in this group were found to be identical. For identities see Autopsy Series above
Marines	54	4	14	Two strains from different individuals were found identical. Of isolations from the same individual: 4th day identical with 6th day 4th day identical with 8th day identical with 11th day 2nd day identical with 8th day 4th day identical with 8th day identical with 15th day 3rd day identical with 7th day, but 14th day was different
Hebrew orphans......	28	5*	16	Two strains were found identical.
Family	6	6	10	All strains were found to be distinct races.

* The results with one serum are given in another table (see original article). The strains were tested with four other sera, but no additional identities were discovered.

DISCUSSION

The results recorded indicate that, under the term *B. influenzae,* we are dealing with a group of organisms which, for practical purposes, is heterogeneous in character as determined by immunological reactions. We have evidences of the existence of small sub-groups. The actual frequency with which identical strains would be encountered could only be determined by the use of many more sera than we have employed. Our own results, however, indicate at least that identical strains would be infrequently encountered.

The existence of a multiplicity of races is advanced as evidence that *B. influenzae* is not the primary etiological agent in epidemic influenza. To controvert this evidence, it would be necessary to prove either that the agglutinin absorption capacity of *B. influenzae* was a characteristic susceptible to rapid change or that, in no instance, have we utilized an actual primary infecting strain for the production of our serums. By analogy with the constancy of the agglutinin absorptive capacity shown by other varieties of bacteria, the former assumption is extremely improbable. The latter assumption that we have failed to employ an actual epidemic strain is rendered very remote especially by the employment of strains isolated early in the disease from groups of cases in close contact.

FURTHER STUDIES ON GROUPING OF INFLUENZA BACILLI WITH SPECIAL REFERENCE TO PERMANENCE OF TYPE IN THE CARRIER

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The first large series of experiments on the relationship of the influenza bacillus to epidemic influenza was carried out at the Research Laboratory under the direction of Park, Williams and Krumwiede. The serological work on grouping was done by Valentine and Cooper. They worked with 171 strains isolated by Williams from different sources during the pandemic of 1918. These strains were tested by the agglutinin absorption method with 25 antisera. The only grouping of strains into types, that could be obtained by this method was as follows: Strains from two individuals among a group of marines infected with influenza were found to be identical. Strains from two children at a children's home also gave identical reactions. As no other definite relationships could be established among the large number of strains tested, the conclusion seemed justified that under the term of *Bacillus influenzae*, just as with the term pneumococcus, we are dealing with a group of heterogeneous organisms. In the case of the influenza bacillus, there is even less suggestion of any dominant types. The existence of a multiplicity of types in cases attacked at the same time and in the same locality, was advanced as evidence against *B. influenzae* being the primary etiological agent in epidemic influenza.

Huntoon and Hannum state that there were no strains among their cultures which did not show relationship, either directly or indirectly through absorption tests. The titer of their serums was rather low for identifying strains by absorption tests. It is extremely probable that with monovalent serums of higher titer many more differences would have been discovered. Moreover, some of their tables show distinct type differences by absorption tests.

Small and Dickson studied ten strains and obtained four type groups from nine strains by subjecting them to agglutinin absorption tests.

Rivers from his investigations thinks that the influenza bacillus can be divided into groups according to their biological characteristics. Thus, he found one definite group of ten strains all of which were similar culturally, all formed indol, all reduced nitrates to nitrites and made blood broth milk slightly acid within forty-eight hours. Besides these he found some strains which produced hemolysis.

In spite of the general agreement in the results of the different investigations, it was thought wise to obtain a large number of new strains and separate them into their immunological types by direct agglutination and agglutinin absorption tests. We utilized about 100 old strains most of which had been isolated by Williams during the pandemic of 1918 and 90 new strains. These last had been isolated by one of us from a series of cases studied by Williams in connection with the recent outbreak of influenza. We had 30 sera, some of which were prepared from the strains of the pandemic, but most from the strains isolated previous to and during the recent outbreak. We tested all our old and new strains with these 30 sera at different times and we state at once that our results are in close agreement with those obtained by Valentine and Cooper. We added three more strains to the Leuchner group of Valentine and Cooper, thus identifying five strains in the Leuchner group (tables 1 and 2 of the original article). A great many other strains were agglutinated to a high titer by heterologous serums but by absorption tests were proven to be alike only in their abundant group agglutinins (table 3 of the original article).

As to the cultures isolated previous to and during the recent epidemic, none except a few contact cases were found to be identical in type, though a great many strains seemed to be closely related as shown by the absorption of a certain amount of agglutinins. We shall, however, speak about these strains from the recent outbreak in more detail later.

In contrast to the cultures from the respiratory tract we found a dominant type of B. influenzae from cases of influenza meningitis. In all, we had seven influenza meningitis cultures, all of which, except one, were isolated in our laboratory (Meningitis Division); of these, two had been isolated a few years before the pandemic, three during the pandemic and one since. One was given to us by Wollstein of The Rockefeller Institute. We developed anti-serums for two of the influenzal meningitis cultures, namely 6741 and 6746. Four of the seven influenzal meningitis cultures were agglutinated by the first serum (6741) and absorbed the agglutinins. None of the remaining three was agglutinated by or absorbed the second serum except the homologous strain. Culture 6746 is therefore, a distinct type. The remaining two cultures were not identical with either of the above types.

Before coming to the discussion of the recent epidemic, we must say a word about the difficulties encountered when working on agglutination tests with *Bacillus influenzae*. The difficulty Wollstein emphasizes, namely, that this organism clumps spontaneously, was with our methods negligible. Ever since the workers in this laboratory began to grow this organism on the chocolate medium and to scrape the growth off in salt solution, instead of washing it off, a homogeneous suspension is the rule. The greatest difficulty in the past has been the capriciousness and unreliability of the influenza bacillus as to its agglutinability. Some time ago, we noticed that the cultures used for animal inoculation and therefore transplanted each day, could be relied upon more surely for their agglutinability than the same cultures kept in stock and transplanted only once a weeek. In order, therefore, to restore to cultures their lost agglutinability we have transplanted them every day for at least a week before using them in a test. In the case of convalescent carriers, we noticed also that when we had a serum ready, the cultures if freshly passed through animals responded much more promptly to agglutination than they had done before or did a little later on. The loss in agglutinability was noticeable within a week's time after isolation. One of our cultures, namely Witt (isolated by Williams from a case of pleurisy following influenza and used by Cecil for his experiments) when tested, failed altogether to agglutinate, but the same culture when passed through the monkey agglutinated beautifully if transplanted every day for one week before doing the tests. As to the serum, if it is of good titer and agglutinates the culture in large clumps, it keeps for long periods without appreciable deterioration in the ice box. We have used such a serum over a period of two years without noting any considerable diminution of its titer.

Our technique is given in the original paper.

Since this paper was published 45 more cultures have been isolated from cases of Influenza Meningitis and we found that the same proportion still holds good, *i. e.*, fifty per cent. of these cultures fall into the dominant type group.

INVESTIGATION OF THE STRAINS FROM INFLUENZA-LIKE CASES IN RETURN OUTBREAK OF THE WINTER, 1920

The object of our studies was twofold: First, to test again the possibility of the constant presence of a dominant type of influenza bacillus which we had failed to find during the pandemic of 1918. If such a type were found, it might be considered the cause of the original pandemic as well as the return outbreak.

Second, to determine the persistence of type characteristics in the strains found in convalescent cases and in normal carriers of the influenza bacillus. If type characteristics were stable, then the finding of many different types in the epidemic cases occurring at about the same time would militate still more strongly against the etiological significance of the influenza bacillus.

With these objects in view, we fished from each case at least 10 colonies and from the selected cases as many as 30 of both small and large influenza-like colonies. Each colony from an Avery medium plate was transplanted on chocolate medium (reaction pH 7.2 to 7.4). Each culture from each separate colony was tested and retested as to its hemoglobinophilic properties by trying it on plain and ascitic agar and studied for its morphology. We utilized the morphology of the hemoglobinophilic organisms to separate the atypical from the typical hemoglobinophilic bacilli.

Atypical hemoglobinophilic bacilli. These are bizarre, long and curved bacilli, often growing out in threads. They seem to correspond to the organisms which Pritchett and Stillman name "the undescribed bacillus X." We found these organisms as the sole hemoglobinophilic organism in 18 cases (some normals and some colds) previous to and during the recent epidemic and in 5 cases coexistent with typical influenza bacilli. Therefore, we felt it worth while to study them more in detail. We found that on the chocolate medium they produced a mucoid growth. They all produced hemolysis, though in varying degree, in tubes with rabbits' washed red cells and in plates with defibrinated horse blood. The hemolytic colonies on superficial examination might be mistaken for hemolytic streptococcus colonies. These organisms do not appreciably ferment glucose as the majority give no reaction and a few show but a trace. They do not produce indol. By means of two sera produced by immunizing rabbits with two different strains, we were able by absorption tests to separate two groups of two and three each; the rest do not belong to either of these two groups and may or may not form many independent groups. Direct agglutination tests are not satisfactory as most of the bacilli clump spontaneously.

Typical hemoglobinophilic bacilli. The morphology of these was such as we usually associate with a typical influenza bacillus; a small slender rod often coccoid in form, later become more irregular and often growing out in threads. It grows profusely on the chocolate medium, between pH 7.2 to 7.4 or 0.2 to 0.3 acid to phenolphthalein (neutral to phenolphathalein before autoclaving).

In order to study the agglutinating characteristics of our separate colonies, we produced over thirty different sera from selected cases.

Rabbits chosen for inoculation weighed between 2000 and 2500 grams. Inoculations were given on three successive days if the condition of the animal allowed and three days rest followed. This process was repeated as often as necessary. An emulsion in saline of 1:40 to 1:10 of the growth on one test tube slant of chocolate agar was used for the initial injection and the amount gradually increased to the growth on two slants. The rabbits on the same culture were found to differ greatly in their resistance and power to form agglutinins. The average time necessary to obtain a serum giving complete agglutination in 1.2500 dilution was seven weeks and the average number of injections was 17.

TABLE 1

INFLUENZAL MENINGITIS GROUP OF CULTURES

DIRECT AGGLUTINATION								AGGLUTINATION AFTER ABSORPTION														
Serum	Culture	100	200	400	600	800	1200	Serum	Culture for absorption	100	200	400	600	800	1200	Serum strain	50	100	200	400	600	1000
6741	6741	+	+	+	+	+	+	6741	6741	−	−	−	−	−	−	6741						
	B. I. W.	+	+	+	+	+	+		B. I. W.	−	−	−	−	−	−	6741	−	−	−	−	−	−
	Z.	+	+	+	+	+	+		Z.	−	−	−	−	−	−	6741	−	−				
	747	+	+	+	+	+	+		747	−	−	−	−	−	−	6741	−	−	−	−	−	−
	6746	±	−	−	−				6746	−	−	−	−	−	−	6741	+	+	+	+	+	+
	6745	±	−	−	−				6745	−	−	−	−	−	−	6741	+	+	+	+	+	+
	6827	±	−	−	−				6827	−	−	−	−	−	−	6741	+	+	+	+	+	+
6746	6746	+	+	+	+	+	+															
	6741	±	−	−	−	−	−															
	Z.	±	−	−	−	−	−															
	6745	±	−	−	−	−	−															
	6827	±	−	−	−	−	−															
	B. I. W.	±	−	−	−	−	−															

Among our most interesting studies of the recent epidemic were those made on a family of five, a mother and four children. They were taken ill with influenza at the same time and were all brought to the hospital. From 10 to 20 colonies were fished from plate cultures of each member of the family. The interesting fact was brought out that the colonies obtained from each member of the family were not all identical with each other; the individuals had probably been carriers of two or more distinct types before the new infection. Homologous serums were prepared with organisms from two members of this family, starting with one colony from an individual in each case. These sera were tested with a number of strains from each case.

To sum up the family table (table 4 of the original article): all except two of the colonies fished from the mother's plates were identical with the serum strain. One of Alfred's colonies was identical with mother's majority strain; the other three were not. One of Theodore's and one of Margaret's colonies were identical with mother's majority strain. All the other strains were different. Four of Alfred's colonies were identical with Alfred's serum strain; three belonged to the atypical hemoglobinophilic bacilli producing hemolysis. One colony was identical with mother's majority strain; two colonies did not belong to any of the groups. One of Alfred's colonies and one of Margaret's colonies, tested by absorption, were identical with Alfred's serum strain.

Outside of this family, in other cases for which we had sera, many colonies were fished and usually showed the same type of organisms. Thus we fished from case 126, 30 colonies, large and small. In case 76, cultures were taken twice a week with a week's interval between; 10 colonies were fished each time. So also three sets of cultures were taken at week intervals from case 39; again 10 colonies were fished each each time. All colonies in each case proved to be identical by direct agglutination and agglutinin absorption with their homologous serums. The same was true of cases 101 and 132.

PERMANENCE OF AGGLUTINATION CHARACTERISTICS DURING DISEASE AND THROUGHOUT CONVALESCENCE

Carriers. Since the pandemic of 1918, Pritchett and Stillman, also Winchell and Stillman found influenza bacilli in 45 per cent. of the throats of convalescents and normal individuals. The duration of the carrier state lasted at times from four to five months. Opie, Friedman and Blake found *Bacillus influenzae* in over 35 per cent. of healthy throats in Camp Funston. They had no way, however, of telling whether the organism remained of the same type or was replaced by another type. The same question is brought up by Bloomfield. In 1918, immediately after the epidemic, we examined convalescent carriers with homologous serums which had been prepared at the time of infection. In one of these cases, still a carrier at the end of the second month, the type remained the same.

From a second case, 50 colonies were fished, 49 of which proved to be identical with the serum strain. One colony was not identical. Two to three weeks later, 10 more colonies were fished from the same throat and none of these were identical with the former 49 colonies, but all were identical with the one strain which was different from the 49. In one case at the Hebrew Orphan Asylum, a culture taken one year later has proved by agglutination and agglutinin absorption tests to be of the same type as that of the year before. But in another case at the same asylum, the culture was of a type different from that of the year before.

In a few of our new cases just before the winter outbreak (for example, case 39 in which cultures were taken once a week for three weeks) the type remained the same by serological tests. The same was true of case 76; two subsequent fishings, one week apart proved to be the same, as well as the organism recovered from the mouse. The cases of accidental infection, reported in paper 5 of these studies, are of importance not only because due to cultures but also because of the proof of the stability of type during the infection.

A most interesting illustration of this is a case of Cecil in his experiments on the infectivity of *Bacillus influenzae.* A volunteer, Loselle was infected with the exudate from the monkey which had been inoculated with one of our pleurisy cultures (Witt). For almost three months he remained a carrier of that same type of organism (see table 5 of the original article.). On the other hand, another of Cecil's cases, Burns, who was a carrier before he was inoculated with culture Witt which had been recovered from the above case, showed on the third day the following among seven colonies: two colonies composed of strains identical with Witt and one unlike Witt; also four colonies which were mixtures of Witt and other types. One week later, no colonies of the Witt type were found. Another of Cecil's cases, Small, inoculated with the Witt strain, showed the culture to be still of the same type after the fifth day.

Crane (Cecil case) was tested culturally at intervals after inoculation with the Witt strain. This strain was found to be present at the end of one hour and also on the third day; but one week later, after a relapse, the culture taken was not of the Witt type when tested by direct agglutination and by agglutinin absorption. The later disease, if due at all to influenza bacilli, was probably due to a new infection.

Cultural types by indol tests. Jordan found that 10 out of 13 strains of *Bacillus influenzae* formed indol. We were able to corroborate his tests by using the paradimethylamido benzaldehyde method that he employed. Out of 42 typical influenza strains, 28 gave indol and 2 more gave traces of indol. None of the atypical hemoglobinophilic bacilli formed indol. We selected our cultures from among different groups in order to see how far the serological tests would correspond with the indol tests and glucose fermentation tests (see table 6 of the original article: meningitis group, Leuchner group, family group).

In the Leuchner group no cultures formed indol. In the meningitis group, three formed indol and one did not although they all were identical serologically; in the family table, all the cultures tested showed indol except those which belonged to the atypical hemoglobinophilic bacilli.

SUMMARY

1. Four out of seven influenzal meningitis strains isolated years apart, were proved by agglutinin absorption tests to be of one type; the remaining three were of different types. From the respiratory cases, five strains from different individuals were obtained which were of the same type. No other groups of more than two members were obtained in agglutination tests with any cultures. This was true of both the original pandemic and also of the new outbreak strains.

2. Atypical hemoglobinophilic bacilli were found in 18 cases (normal individuals and cases of cold) and in five cases these were found in coexistence with typical influenza bacilli. These atypical organisms produced hemolysis, did not form indol and did not ferment glucose.

3. About 75 per cent of typical influenza bacilli form indol; from 50 to 60 per cent. ferment glucose somewhat irregularly and not energetically.

4. The indol reactions did not correspond fully among the meningitis group of four serologically identical strains. Of these, three formed indol—one did not. In the family group, though the strains were not all identical serologically, all except the atypical hemoglobinophilic colonies formed indol. In general, it was found that strains having the same immunological characteristics had the same cultural reactions, but there were some exceptions.

5. Many colonies were fished from each of a number of selected cases and tested with homologous and heterologous serums. In a few cases as many as 20 or 30 colonies from each case were tested with the homologous serum prepared from one of these colonies. In most cases the fishings of influenza bacilli proved to be of one type; at least one type greatly predominated. But in a minority of instances, the colonies from the same case were not in the same type as proved by agglutination absorption tests.

6. In carriers or where the infection was mild, more than one type variety of the influenza bacillus were apt to be found in the same case.

7. In several convalescent carriers, tests were made during periods of from several weeks to three months. These resulted in finding in all but one, the original type of the influenza bacillus. In one case, tests made one year apart proved the original type present.

For bibliography see the original article.

ANNOTATION: The study of influenza bacilli isolated from cases of meningitis has been continued.

As mentioned above, four strains out of seven fell into one group by agglutination and absorption tests. For this work we used a monovalent serum from rabbits immunized against the oldest strain of that group; namely, Bacillus influenzae w. Since that time we have obtained, from Dr. Neal, influenza cultures isolated from cases of meningitis as follows:

1922	13 cultures
1923	6 cultures
1924	5 cultures
1925	3 cultures
1926	6 cultures
1927	2 cultures
Total	35 cultures

About 50% of these cultures continue to fall into the same group which is now designated as the "Predominating Influenza Meningitis" group or Type I. Two horses have been immunized with the strains of this predominating group and the serum used therapeutically in cases of influenzal meningitis.

ACCIDENTAL INOCULATION OF INFLUENZA BACILLI ON THE MUCOUS MEMBRANES OF HEALTHY PERSONS WITH DEVELOPMENT OF INFECTION IN AT LEAST ONE. PERSISTENCE OF TYPE CHARACTERISTICS OF THE BACILLI.

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The three accidental inoculations of influenza bacilli (eighteen hour cultures on chocolate agar), are of interest from the fact that infection developed in one case on the fourth day; possibly in one case on the fourteenth day and not at all in the third case. All three persons, however, became persistent carriers of the bacilli and in all cases the strains held their agglutination characteristics unchanged during the period of the tests. The details of three accidental inoculations are as follows:

Case I. It is difficult to judge whether this case was really one of infection from inoculation with the Pfeiffer bacillus or not because of the long period of incubation. Dr. H. accidentally inoculated her throat with the Lee culture, an autopsy strain from an epidemic case, while expelling a culture suspension from a syringe. The culture was an eighteen-hour growth on "chocolate blood" mixed with human serum. It happens that some time before the accident a culture from Dr. H. had been made and gave no influenza bacilli. No symptoms developed for two weeks when she developed a temperature of 101, prostration and general pains. She was confined to bed. On her return, plate cultures made from the nasopharynx showed abundant influenza bacilli.

Ten colonies were fished from a pharyngeal culture.

All isolations were alike and identical in type with the accident strain. The direct agglutinations are given in table 1. Two of the strains were

TABLE 1

AGGLUTINATION OF H STRAINS (EXAMINATION I) BY LEE SERUM

STRAINS	SERUM DILUTIONS						CONTROL
	1:50	1:100	1:200	1:400	1:700	1:1000	
H-I-1	+	+	+	±	×	−	−
H-I-2	+	+	+	±	×	−	−
H-I-3	+	+	+	±	×	−	−
H-I-4	+	+	+	±	×	−	−
H-I-5	+	+	+	±	×	−	−
H-I-6	+	+	+	±	×	−	−
H-I-7	+	+	+	±	×	−	−
H-I-8	+	+	+	±	×	−	−
H-I-9	+	+	+	±	×	−	−
H-I-10	±	+	+	±	×	−	−
Lee	+	+	+	±	×	−	−

+ = Complete agglutination.
± = Marked agglutination.
× = All reactions weaker than those above.
− = No reaction.

further investigated by agglutinin absorption tests with the Lee serum (table 2). The identity of the Lee cultures and the H. strains was confirmed. Sixty-eight days after the accident 11 more strains were isolated from a nasopharyngeal culture. These were found identical with the previous autopsy and H. strains (table 3).

TABLE 2

AGGLUTININ ABSORPTION TESTS WITH H STRAINS (EXAMINATION I) AND LEE SERUM

STRAINS	AGGLUTINATION BEFORE ABSORPTION						ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION												CONTROL
								Absorbing strain						Serum strain						
	1:50	1:100	1:200	1:400	1:700	1:1000		1:50	1:100	1:200	1:400	1:700	1:1000	1:50	1:100	1:200	1:400	1:700	1:1000	
H-I-3	+	+	+	±	×	−	H-I-3	−	−	−	−	−	−	−	−	−	−	−	−	−
H-I-7	+	+	+	±	×	−	H-I-7	−	−	−	−	−	−	−	−	−	−	−	−	−
Lee	+	+	+	±	×	−	Lee	−	−	−	−	−	−	−	−	−	−	−	−	−

Case II. Miss B. accidentally sucked into her mouth some salt solution suspension of an eighteen-hour culture on blood agar. She developed symptoms of a pharyngeal infection on the fourth day. There was a slight rise of temperature and moderate prostration. On her return to work nasopharyngeal cultures yielded influenza bacilli identical in type with those in

TABLE 3

AGGLUTINATION OF H STRAINS (EXAMINATION II) BY LEE SERUM

STRAINS	SERUM DILUTIONS						CONTROL
	1:50	1:100	1:200	1:400	1:700	1:1000	
H-II-1	+	+	+	±	×	−	−
H-II-2	+	+	±	±	×	−	−
H-II-3	+	+	+	±	×	−	−
H-II-4	+	+	+	±	×	−	−
H-II-5	+	+	+	±	×	−	−
H-II-6	+	+	+	±	×	−	−
H-II-7	+	+	+	±	×	−	−
H-II-8	+	+	+	±	×	−	−
H-II-9	+	+	+	±	×	−	−
H-II-10	+	+	+	±	×	−	−
H-II-11	+	+	+	±	×	−	−
Lee	+	+	+	±	×	−	−

the Holmes culture (table 4). Holmes was one of a group of marines among whom influenza developed some weeks before.

TABLE 4

AGGLUTININ ABSORPTION TEST WITH B STRAIN AND HOLMES SERUM

STRAINS	AGGLUTINATION BEFORE ABSORPTION						ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION												
								Absorbing strain						Serum strain						CONTROL
	1:100	1:200	1:400	1:600	1:800	1:1000		1:100	1:200	1:400	1:600	1:800	1:1000	1:100	1:200	1:400	1:600	1:800	1:1000	
B	+	+	+	+	+	+	B	–	–	–	–	–	–	–	–	–	–	–	–	–
Holmes	+	+	+	+	+	+	Holmes	–	–	–	–	–	–	–	–	–	–	–	–	–

Case III. Miss R. was accidentally infected with Marine 3 culture, a fourth day case strain isolated from the nasopharynx. She received infection in the same manner as Dr. H. Miss R. was not ill after the accident. Twelve strains isolated from plate cultures made twenty-eight days after the accident were all alike and were identical with the accident strain (tables 5 and 6).

Case IV. Miss C was accidentally exposed in a manner similar to Miss R. and Dr. H. but she neither developed symptoms nor became a carrier.

TABLE 5

AGGLUTINATION OF R STRAINS BY MARINE 3 SERUM

STRAINS	SERUM DILUTIONS						CONTROL
	1:50	1:100	1:200	1:400	1:700	1:1000	
R-1	+	+	+	+	+	±	–
R-2	+	+	+	+	+	±	–
R-3	+	+	+	+	+	±	–
R-4	+	+	+	+	+	±	–
R-5	+	+	+	+	+	±	–
R-6	+	+	+	+	+	±	–
R-7	+	+	+	+	+	±	–
R-8	+	+	+	+	±	±	–
R-9	+	+	+	+	+	±	–
R-10	+	+	+	+	+	±	–
R-11	+	+	+	+	+	±	–
R-12	+	+	+	+	+	±	–
Marine 3	+	+	+	+	+	±	–

TABLE 6

AGGLUTININ ABSORPTION TESTS WITH R STRAINS AND MARINE 3 SERUM

STRAINS	AGGLUTINATION BEFORE ABSORPTION						ABSORBED BY STRAINS	AGGLUTINATION AFTER ABSORPTION												CONTROL
								Absorbing strain						Serum strain						
	1:50	1:100	1:200	1:400	1:700	1:1000		1:50	1:100	1:200	1:400	1:700	1:1000	1:50	1:100	1:200	1:400	1:700	1:1000	
R-3	+	+	+	+	+	±	R-3	–	–	–	–	–	–	–	–	–	–	–	–	–
R-11	+	+	+	+	+	±	R-11	–	–	–	–	–	–	–	–	–	–	–	–	–
Marine 3	+	+	+	+	+	±	Marine 3	–	–	–	–	–	–	–	–	–	–	–	–	–

CONCLUSIONS

1. Freshly isolated Pfeiffer bacilli are capable of causing infection when transferred in great numbers to susceptible mucous membranes.

2. Strains recovered from infected persons at varying intervals after the accidental inoculation, revealed agglutinative characteristics identical with those of the strains which had caused the infection. The varying periods cover the time from shortly after infection to convalescence. This is strong evidence that a strain responsible for an infection would reveal identical characteristics during infection and convalescence in the epidemic cases due to its invasion. If the epidemic of influenza were due to the Pfeiffer bacillus, we would expect the dominant strains to have similar type characteristics. The fact that just the opposite condition exists is strong proof against the primary etiological importance of the Pfeiffer bacillus in epidemic influenza.

REPORT ON THE PROPHYLACTIC VACCINATION OF 1536 PERSONS AGAINST ACUTE RESPIRATORY DISEASES, 1919-1920

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Anna I. Von Sholly and William H. Park

The reason for the investigation upon which this report is based, was to attempt to clear up, if possible, the status of a vaccine made of the mixed organisms which predominate in respiratory diseases, used as a prophylactic agent in epidemic "grippe" and acute respiratory diseases in general. Reports on vaccine prophylaxis are only too plentiful both for vaccine made of pure *Bacillus influenzae* and for a vaccine made of various mixtures of the important microbes found in the respiratory tract. The chief criticism to the recognition of their claims is that they were tested out during the late pandemic of "grippe" either close to or after the peak of the epidemic, at a time when it was impossible to sort out cases into convincing statistical groups on account of the unknown factor of natural or inherited immunity which plays an important and as yet little charted part in epidemiology. Because, too, of this unknown denominator of natural immunity, adequate control groups, for scientific comparison with the inoculated groups, during the epidemic period, were not obtainable.

Our aim, then, in this study, was to inoculate prophylactically a group of people well in advance of any anticipated recurrence of epidemic grippe so that whatever immunity might be stimulated by this method would be already established before exposure to the disease. Moreover, we wished to learn what effect a vaccine made of the predominant organisms of the acute respiratory diseases would have in inhibiting or modifying these diseases. We aimed, too, to have for comparison an uninoculated control group as similar as possible to the group inoculated.

The work presented was done between September 30, 1919, and April 3, 1920, among the general employees of the Metropolitan Life Insurance Company's Office, New York City, under the direction of the Research Laboratory of the New York City Health Department, coöperating with the officers of the Metropolitan Life Insurance Company. This company gave the services of its nursing and clerical forces and defrayed the cost of the vaccine. The groups under observation consisted of men and women, all volunteers, of varying ages. In all, there were 1536 people who were inoculated, of whom 1293 received three inoculations, 95, two inoculations, and 148 only one inoculation. As a control group 3025 persons of similar ages and both sexes of the same company, were under continuous observation. Only about 13 per cent. of the original inoculated group and 14 per cent. of the uninoculated dropped out during the six months of investigation.

The personal history, home conditions and habits of the entire group as given in the original form filled out by each volunteer at the beginning of our work, summarized in percentages taken from 1500 histories selected at random shows similarity between the two subgroups; i.e., inoculated and uninoculated.

Method of Following the Groups

On entering the group, each person filled out a form, which was kept under his or her name in a filing cabinet. All subsequent records of each person were attached to this form as they were collected. That we might not slip up in following the course of each one's health, we imposed a series of checks in the collection of the material. A form was given each person to fill out and return when he or she was suffering from any acute respiratory disease. Still another form was given section heads who were made responsible for their weekly return to us with the names and condition of those in the section suffering from "colds." In addition, copies were made daily from the dispensary records of all persons who had reported for treatment for any respiratory disease during that same day. For several months, many of these people were seen personally by one of us (von Sholly) in order to obtain material for culture work. Furthermore, from the Medical Welfare Bureau which has supervision over all employees who are absent for illness, we obtained daily reports, with diagnoses of the disease and length of absence. Pneumonia cases were investigated to confirm the diagnosis. All the facts obtained were inscribed daily on the records and filed away. To indicate the extent of the statistical work, we may state that we had one doctor, one nurse, two whole time clerks, two part time clerks for a fairly long period, and one laboratory assistant at work daily, for more than six months. We do not consider our records perfect. They could not be so, where one had to deal with a freely moving volunteer group of varying degrees of intelligence and coöperation. We hope, however, that they are better than many that have been offered. Undoubtedly we did not get a record of every "cold" experienced in the group but feel confident that whatever errors have crept in were true of both groups, inoculated and not inoculated, and that, with the check of the absentee and dispensary reports, only the mildest affections requiring slight treatment and no absence are not recorded. The individual diagnoses, too, may not be strictly accurate. Some were self diagnosed (checked as far as possible by the Company's physician). Furthermore, the diagnoses of outside physicians may not conform absolutely to our nomenclature. By grouping the infections, however, into upper and lower respiratory diseases, we think they are fairly accurate—sufficiently so for the purposes of this investigation.

The injections of vaccine were given at weekly intervals during the end of September, 1919, and during the first three weeks of October, 1919. Two vaccines[1] were used. The larger group of persons numbering 1412, were given vaccine *L*, a saline suspension sterilized by heat and made up of:

	million per cc.
B. influenzae	1000
Streptococcus hem	1000
Streptococcus vir	1000
Pneumococcus 1	2000
Pneumococcus 2	2000
Pneumococcus 3	1000

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[1] These formulae were selected by a committee composed of Dr. Park of the Department of Health, City of New York, Dr. Rosenau of Harvard, Dr. McCoy of the United States Public Health Service, and Professor Jordan of Chicago University.

The smaller group, numbering 124, were given a vaccine *R,* a vaccine similar in composition to that given by Rosenow of the Mayo Clinic. It consisted of:

	million per cc.
Pneumococci type 1 (10 per cent.), 2 (14 per cent.), 3 (6 per cent.)	1500
Pneumococci, group 4 and allied green producing Diplostreptococci	1500
Hemolytic streptococci	1000
Staphylococci (aureus)	500
B. influenzae	500

We kept a record of the reactions to the vaccines given, partly for general scientific knowledge and partly for the purpose of finding out the practicability of group vaccination for a commercial company from the point of view of loss of services following and due to inoculations. The injections were given subcutaneously in the deltoid region. The first dose was 0.5 cc. and the second and third were 1 cc. each.

There were, following the first injection, mild local reactions (area of redness not more than a silver dollar in size, and very slight soreness) among 63.8 per cent. of the group; moderate local reactions, (area of redness from size of a silver dollar to size of hand and slight soreness) in 11.8 per cent. of group; marked local reactions, (anything larger than moderate) in 3.3 per cent. of the group and no reaction at all in 21 per cent. Following the second injection (double the dose) mild local reactions were observed in 63.2 per cent. of the group, moderate in 21.4 per cent., marked in 2.7 per cent. and no reactions in 12.7 per cent. Following the third injection, mild local reactions were seen in 60.6 per cent., moderate in 9.7 per cent., marked in 4.1 per cent., and none in 25.5 per cent.

Of the general reactions, the mild ones, consisting of headaches, slight dizziness, nausea or general malaise were reported after the first injection, in 19.4 per cent. of the group; after the second injection in 27 per cent. and after the third in 23.7 per cent. The marked reactions, chills or chilly feelings, fever, general pains, or mild prostration, after the first injection, were observed in 3.1 per cent.; after the second, in 3.2 per cent. and after the third, among 2.6 per cent. Only 26.2 per cent. of the total inoculated gave general reactions. These figures show that the largest number of reactions, as was to be anticipated, followed the second injection and that the severe reactions were comparatively few. There were no really alarming reactions. The details of the reactions of the vaccines are given in table 1 in the original article.

That some people should take advantage of the inoculations to remain at home would not be at all surprising, especially, where there is an illness insurance. If we give everybody the benefit of the doubt and count all absences immediately following vaccination as due to it, there were in all 87 persons who were absent from work anywhere from a half day to 24 days. The total days absence of this group was 181½ days, or 2 days and 42 minutes for each one. The average absence per person for the total inoculated group, 1536 persons, taking the working day as 8 hours, was about 56 minutes. We may compare these percentages with the absences due to respiratory diseases during the six months observation period. The uninoculated lost 8172 days or an average of 2.2 days per person and the inoculated lost 2456 days or an average of 1.59 days per person during the same interval. If we add the average of the inoculated persons' absence due to respiratory disease to the average absence credited to inoculations, we still find a gain of a good half working day per person in favor of the inoculated. It is fair also to assume that if the custom of annual vaccinations were established, absences due to inoculations would diminish.

A tabulation was made to learn whether there was any demonstrable relationship between past respiratory diseases, such as pneumonia, bronchitis, grippe, etc., and the character and severity of the reactions from the inoculations. No especial susceptibility to vaccines can be established for these diseases. The same was true for the "allergic" diseases, asthma, hay fever, etc., but the numbers in our group are probably too small to show anything.

Respiratory Affections Among the Vaccinated and Unvaccinated During the Six Months Observation Period October 1, 1919, To April 3, 1920

There were 1327 completed records of inoculated persons under continuous observation during the above period and 3025 similar records of the uninoculated. Among the inoculated, 13.7 per cent. give a history of *no respiratory affections* during this time. Among the non-inoculated 29.77 per cent. give a similar history.

The respiratory infections which occurred in each group and their severity are tabulated in table 3 of the original article.

One might infer from the tabulated figures that it were wiser not to be inoculated. One must remember, however, that the observed groups were volunteers. Naturally, those volunteering for inoculation might be supposed to be the more susceptible to respiratory diseases, hoping for relief from the inoculations. And, in fact, our summaries of the original questionnaire show that 21.6 per cent of the non-inoculated were comparatively free from respiratory troubles in their past, as against 10.5 per cent of those who submitted to inoculations.

It is probably fair to conclude that, considering the results as a whole, the kind of vaccine use by us, in the manner and dosage indicated, had very little specific influence in preventing or causing respiratory diseases other than pneumonia.

Pneumonia

The difference of pneumonia incidence, however, is marked in the two groups.

The inoculated show 1 case (0.075 per cent) against 11 cases (0.36 per cent) among the non-inoculated, or about five times as many pneumonias among the non-inoculated.

It is true that the number of cases of pneumonia occurring in the group is so small that the apparently favorable results of vaccination may be due to chance, nevertheless, it would seem fair to make some claims for the efficacy of a typed pneumo-streptococcus vaccine as a preventive of pneumonia. The one case of pneumonia which occurred in the inoculated group, was a girl who had had only one injection of the vaccine on October 2, 1919. She suffered from influenza and broncho-pneumonia March 23, 1920, more than five months after vaccination. She was absent from her work just one month. She had had an acute bronchitis (absent 20 days) in January, 1920. Lister in South Africa does not claim much immunity resulting from the pneumococcus vaccine for more than four to six months. After our investigation had been closed, two more cases of pneumonia, were reported among the inoculated. Both occurred in May, 1920, about seven months after the vaccinations. One of these patients had had two and the other three injections.

Of the 11 cases of pneumonia occurring among the non-inoculated, before April 3, 1920, seven were lobar in type and four were broncho-pneumonia. Two of the lobar pneumonias and two of the broncho-pneumonias were said to complicate grippe. One case of lobar pneumonia (primary) died after three days illness. In May 1920, a twelfth pneumonia was reported also among the non-inoculated. If we count these later cases, the percentage was still twice as high for pneumonia among the non-inoculated persons.

GRIPPE CASES

One of the most important phases of our investigation was the completion of the vaccinations about three months before the brief flare up of grippe in January and February, 1920. In the inoculated group, there were 232 grippe cases[2] during the six months, most of them occurring during January and February. These made up 17.8 *per cent.* of the inoculated group. Of these cases 49 (21.1 per cent.) had had grippe in the pandemic of the previous year (1918-1919), 191 of the 232 had been inoculated three times (in October, 1919), 14 had received only two injections and 26 only one injection.

Among the uninoculated, there were 494 cases of grippe, excluding the pneumonias, reported in the period of observation. These were 16.3 *per cent.* of the group. Eighty-eight persons (17.8 *per cent.*) had had influenza also during the previous year. In comparing these groups, we find very little difference in their respective degrees of immunity to what was considered as a recurrent outbreak of influenza.

Looking at these same figures from the point of view of the 1918-1919 epidemic forward to the 1920 return, the results are practically the same. Of the group reporting grippe in 1918-1919, 12.2 *per cent.* had a second attack in 1920 after inoculation, and 13.1 *per cent.* had a second attack, although not inoculated.

Most of the grippe cases of 1920 were comparatively mild. The inoculated averaged 7.2 days absence per person, and the non-inoculated averaged 9.2 days per person. The difference can be accounted for only to a slight degree by the fact that the pneumonias occurred more frequently among the non-inoculated.

SEVERITY OF ILLNESS

It was a rather difficult matter to gauge accurately the severity of the various infections. One had to rely partly on the personal opinion of the patient. However, we made an attempt to judge by an arbitrary classification, as follows: Illnesses were considered severe that showed (1) fever over 102°, (2) confinement in bed for four days or more, (3) marked specific clinical symptoms, (4) some prostration. Moderate illnesses were those with (1) fever 100° to 102°, (2) confinement in bed or at home two to four days, (3) moderately severe specific clinical symptoms, (4) malaise. Mild infections were those with (1) a temperature less than 100°, (2) absence less than two days, (3) moderate or mild specific clinical symptoms, (4) slight or no malaise. Our statistics for severity of disease were taken from the absentee reports which, obviously, were the less difficult to judge, since all absentees are visited in their homes by the Company's nurse if they are away more than three days, and all are seen by a nurse or the Company's doctor on their return to work.

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[2] After these figures were obtained, there were a few doubtful cases subtracted—which however made practically no difference in the relative percentages.

In the inoculated group, 445 (30.8 per cent.) were absent during the period of October 30, 1919 to April 3, 1920. In the not inoculated group, 1235 (36.1 *per cent.*) were absent. If we divide these absentees into groups according to the degree of severity of their illness, we find 70.8 *per cent.* of both the inoculated and the uninoculated fall into the mild class; 26.7 *per cent.* of the inoculated against 25.7 *per cent.* of non-inoculated fall into the moderate class, and 2.4 *per cent.* of the inoculated against 3.4 *per cent.* of the uninoculated fall into the severe class.

From the examination of the foregoing stastistics, we may draw contrary conclusions depending upon whichever part of the report we lay more stress. If we compare the incidence of the respiratory affections among the inoculated and the uninoculated, excluding pneumonia, and if we consider the severity of these respiratory affections, we may assert that the vaccines were of little or no appreciable value. If, on the other hand, we focus our attention on the figures that show a somewhat higher susceptibility to respiratory diseases among the vaccinated in their past histories; a larger group absence for respiratory diseases among the not vaccinated and a longer average absence per person for attacks of "grippe" in the not inoculated group, we may conclude that the vaccines had a beneficial influence. On the whole, balancing both sides, our evidence does not make a strong case in favor of the vaccines given us as a prophylactic agent against acute respiratory diseases—pneumonia alone excepted. Our work tends to confirm the conclusions of Lister and Cecil and Austin on the value of a typed pneumococcus vaccine for pneumonia. It points to the importance of further research along this line.

TABLE 1

SEVERITY OF RESPIRATORY DISEASES AMONG ABSENTEES ON ACCOUNT OF SICKNESS

MILD AFFECTIONS	MODERATE AFFECTIONS	SEVERE AFFECTIONS	TOTAL PERSONS	PER CENT WHOLE GROUPS ABSENT FOR RESPIRATORY SICKNESS
Inoculated group				
315 (70.8%)	119 (26.7%)	11 (2.47%)	445 (100%)	30.8%
Not inoculated group				
874 (70.8%)	317 (25.7%)	42 (3.4%)	1235 (100%)	36.1%

Thanks are due for the help and courtesy extended to us by the staff of the medical and statistical departments of the Metropolitan Life Insurance company.

For bibliography see the original article.

STUDIES ON VACCINE THERAPY FOR PNEUMONIA AFTER 1920.

Anna I. Von Sholly

In April, 1921, the New York City Health Department, began to try out at one of the large State Hospitals the value of a combined Type I, II and III pneumococcic vaccine as a prophylactic. This trial was continued for a period of three years. The average daily census of the inmates of this institution was about 6,800 persons. One half were inoculated and the other half held as controls. The aged, feeble and tuberculous were excluded, so that a little less than 3000 persons were included in each group, the inoculated and the controls respectively. Several series of re-inoculations were given, some groups after six months intervals and other groups after one year intervals. The total dosage (divided into three injections at 5-7 day periods) was 30 billion in 1921-22, 22½ billion, in 1922-23 and 36 billion in 1923-24.

During the three years' study there were 12 cases of lobar pneumonia among the inoculated with 4 deaths and a similar number (12) among the unvaccinated controls with 8 deaths. Unfortunately however, on account of the difficulty of obtaining the co-operation of the patients, we were able to type the sputums of only three cases among the inoculated and only 5 among the controls. The three cases typed in the inoculated group (all of whom recovered) were two cases of Type III, (inoculated 7 weeks and 5 months respectively, previous to their illness), and one case of Type II inoculated 6 weeks before. None of the fatal cases in the inoculated group was typed.

The five typed cases among the control group were one case Type II, one case Type III and 3 cases Type IV. Three died, one of each of the above type cases.

The intervals that elapsed between the last series of inoculations and the attack of pneumonia among the inoculated who recovered were as follows:— three, within 5-7 weeks after the last series of inoculations, one, 3 months after, one 4 months after and three 5 months after. The four that died had received their last series, 3, 5, 6 and 7 months previously.

Terminal pneumonias and acute broncho-pneumonias are not included in the above report. According to Lister and Cecil and Austen, Type I, II and III pneumonias should not have occurred after inoculation. While our results are not as brilliant as those obtained by these investigators, they are not wholly discouraging. The incidence of pneumonia and the occurrence of the fixed type pneumonias were apparently not lessened by previous inoculations with the pneumococci, the mortality, however, seems to have been influenced very decided for the better.

STUDIES ON ACUTE RESPIRATORY INFECTIONS

A Study of the Cultural and Serological Relationship of Hemolytic Streptococci Isolated from Inflammatory Conditions Of the Respiratory Tract[1]

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EUGENIA VALENTINE AND LUCY MISHULOW

This study is part of the general investigation of the diseases of the respiratory tract. The purpose of this study was to determine whether or not a dominant variety of hemolytic streptococcus could be isolated from the cases of acute colds and influenza studied.

The rôle played by the hemolytic streptococcus in a considerable percentage of the fatal pneumonias in 1918 has stimulated many investigators to study the incidence of this organism in the secretions of the nose, throat and in the sputum of normal persons and in conditions of respiratory disease. They also studied the fermentative reactions in an attempt to correlate the fermentative types with the character of the respiratory condition.

The work of these investigators is of interest primarily in that it indicates that the streptococcus hemolyticus is frequently present in the throat, especially if the tonsils be large and cryptic. As to the correlation of definite fermentative types with the conditions in which they were found, the results are not altogether concordant. These results bear only indirectly on our problem, as the fermentative reactions give only broad primary groups, which groups are further divisible by immunological reactions.

Several investigators have studied the immunological reactions of the streptococci isolated from respiratory diseases. Most noteworthy is the work of Dochez, Avery and Lancefield who studied the immunological relationship of hemolytic streptococci especially from cases of pneumonia, many of which were secondary to measles. The material was obtained mainly from the military establishments about Fort Houston. These authors added to the evidence already available that fermentative differences are strongly indicative of immunological differences, whereas fermentative similarity is not necessarily parallel by immunological similarity. The earlier studies, especially those of septic sore throat (Smith and Brown, Krumwiede and Valentine) had indicated this.

In our attempt to find a dominant strain of streptococcus hemolyticus in this series of cases, the plan was to study first the general biological characteristics in order to determine what groups were separable by fermentative and hemolytic reactions. The members of these groups were then studied immunologically by agglutination, and where strains were seemingly alike, agglutinin absorption was employed as the final criterion of identity or non-identity.

The cultures studied were obtained from the nose, naso-pharynx or throat in acute colds, rhinitis and pharyngitis, and from the more severe influenzal conditions. A few pleurisy and mastoid cases are included, as well as normal controls. In many instances several examinations were made from the same case both during illness and after recovery.

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[1] One of a series of studies carried out under the direction of William H. Park, Anna W. Williams and Charles Krumwiede. The previous studies in this series were reported in the Journal of Immunology, vi, no. 1, January, 1921. This investigation was made possible by a grant of money from the Metropolitan Life Insurance influenza fund for a part of the expense.

The method of isolation of the organisms for this study was as follows:

A sterile cotton swab was rubbed over the surface of the mucous membrane of the nose, nasopharynx or tonsils and the material thus collected was rubbed up in broth which was used for "shake" pour blood plates. Some of this broth suspension was also inoculated intraperitoneally into white mice. If these succumbed, pour blood plates were made from the heart's blood. Material obtained by nasal irrigation with Ringer's solution was also used for pour blood plates. The blood plates were incubated for forty-eight hours and the colonies showing definite macroscopic zones of hemolysis were fished for further study. The strains were kept on blood-streaked agar and transferred fortnightly pending the comparative tests.

The data as to the individual case, the type of disease, the source of the culture, how obtained (from mouse or direct plate), and the number of examinations made on the patient are given in tables 1 to 6 of the original article. The scheme used for numbering the cultures is exemplified by the following complex numbers, 112, II, 4. The first number refers to the case from which material was obtained for bacteriological examinations. As hemolytic streptococci were frequently not obtained there is no sequence in these numbers. The second or Roman numeral refers to the different specimens taken at different intervals from the same patient. In the majority of cases only one specimen was taken. In one specimen from a case, hemolytic streptococci might be absent but present in a subsequent specimen, which accounts for the absence of some numbers. The last (Arabic) number refers to the strains isolated from the material. Omitted strain numbers are accounted for by loss of cultures or by failure to show sufficient hemolysis on subsequent tests to warrant inclusion in this series.

Hemolysis Tests

The first reaction studied was hemolytic activity. All of our strains were tested by both the plate and tube methods. The pour blood plates were observed according to the directions given by Brown. The changes in the blood plate are given according to his terminology. This reaction is discussed more fully in another paper of this series. The tube method employed was also that of Brown.

As noted above, the primary selection of streptococci as hemolytic was based on the presence of macroscopically visible clearing in the original pour blood plates. On replating the cultures and observing them after forty-eight hours incubation followed by forty-eight hours on ice, it was evident that we were dealing with three types of colonies as regards hemolysis (Brown). The colonies of the alpha type (definitely green and showing only slight if any clearing) were discarded. This type is considered in another article in this series. The remaining cultures fell in either the "alpha prime" or "beta" groups. The "alpha prime" type is characterized by colonies having a distinct zone of clearing, varying in diameter from 2 to 5 mm., at the end of forty-eight hour incubation. When examined under the microscope, the red blood cells are intact immediately around the colony, with the zone of clearing beyond either complete or partial. The "beta" type at the end of forty-eight hours incubation in pour blood plates shows a complete destruction of the red blood cells about the colony and in the area beyond, as determined by microscopic examination. In general the outer edge of the hemolytic zone is more clear cut than in the "alpha prime" types.

The test tube method has added no information. As is evident from the table there is no absolute correlation in the amount of hemolysis in the test tube and the reaction on the blood plate, except that the beta types as a group show a greater ability to hemolyze red blood cells in the test tube than the alpha prime types. The reaction in the blood plate, observed under the conditions advised by Brown, not only gives all the information that the tube method gives but also allows of a further separation into the two fairly distinct varieties (alpha prime and beta). The difference between the two types of colonies may not be very marked in some instances. Additional comparative work as reported in a paper in this series indicates that the slight differences in hemolysis are due essentially to the action of two distinct hemolytic substances. If, as it seems, we are dealing with two different hemolytic substances, that is, with a difference in biologic action in the alpha prime and beta types we are then justified in utilizing this distinction as a primary basis for subdivision.

One group of cultures 168, V, 1, and W, I, 1, 2, 3 and 4 (table 3 and table 7 of the original article) is distinctive.[2] With the blood plate method they give a beta type of hemolysis in that there is a complete hemolysis of the cells about the colony but the surrounding zone is comparatively narrow. With the test tube method these cultures give a slight hemolysis at most and frequently are negative.

Two factors enter probably into this apparent discrepancy. These cultures grow feebly in broth at best and it is probable that, at any one time, the accumulated amount of hemolysin is small because production does not outpace deterioration. In the blood plate, however, the hemolysin acts as it is formed and the resultant hemolysis on the plate is a truer index as to the nature of these strains. More detailed comparative data are given in another article in this series.

The above mentioned cultures, 168, V, 1 and W, I, 1, 2, 3, 4, showed an added peculiarity on the blood agar plate. Their colonies were characterized by a distinctive rosette shape. This characteristic remained constant on further cultivation. Such a colony would be classed by Brown as complex. However, he described no complex colonies of the beta type.

Another peculiar colony morphology was presented by a group of cultures of which serum strain 77 is the prototype. These colonies after twenty-four hours incubation were large, raised, mucoid and glistening; after forty-eight hours they had flattened and dried and when touched with the platinum wire appeared brittle. On further cultivation there was a marked tendency of these colonies to lose this peculiar characteristic. Nine cases yielded colonies which showed this colony morphology. The results of the tests on these strains are summarized in table 8 of the original article.

FERMENTATION TESTS

The next reaction considered was the fermentation of lactose, salicin, mannite, raffinose, saccharose. A highly satisfactory basic medium contained 0.5 per cent. of agar neutral to the andrade indicator (used in a 1 per cent. amount). To this medium was added 1 per cent. of the test substance. This semi-solid medium was inoculated by means of narrow pipette, containing a fresh eighteen hour broth culture. The pipette was thrust down into the medium. and then withdrawn, 0.2 cc. being allowed to flow from the pipette during the withdrawal. The tubes were incubated at 37°C. and observations were made on the first, third and seventh days. The maximum degree of color change and the days on which the maximum color first appeared is recorded in the table.

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[2] See Williams, Unneberg, Goldman and Hussey (11) for original description of these cultures under the terms "Witt" and "Witt like."

In subdividing the strains on the basis of hemolysis and fermentative reactions, Brown's classification has been utilized as far as our data allowed. It will be noted that inulin was not employed. We omitted this carbohydrate because the hemolytic streptococcus ferments it so frequently. This being the case the use of the Brown nomenclature seemed justified notwithstanding the omission of this test.

A moderate degree of variation has been noted in the fermentation tests. This variation has been limited to instances where the avidity for a fermentable substance was not great. That is, a slight positive result might on a successive test be negative or vice versa. In these instances the positive findings were recorded, as it was found that when a large number of tests were made on a given strain more positive results were obtained.

In general the fermentation reactions of the beta types were more constant and they showed a greater fermentative capacity, in that positive reactions were stronger and appeared more promptly. Occasional exceptions were encountered. The most noteworthy example is seen with all of the strains of case 77 and with strains 72 I, 3. The slight reaction with lactose was uniformly elicited and in itself warrants the separation of these cultures from the other pyogenes varieties. Such a peculiarly low but constant avidity for a fermentable substance has apparently a definite classificatory significance among streptococci. As will be noted later these strains are serologically alike. We had encountered this peculiar low avidity for one sugar in cultures obtained during an investigation of an epidemic of septic sore throat. In that instance the epidemic strain was separable from other strains by a low avidity for salicin.

In table 9 of the original article the strains from the same case which showed differences in biological reaction are compiled. The instances where all the strains from the same case are alike in so far as studied, are easily seen in the preceding tables. Comparison shows how relatively few cases yielded dissimilar strains. This is probably no index as to number of varieties present in a single case because the number of strains obtained from each case was relatively few.

Determination of Final Hydrogen Ion Concentration in Dextrose Broth

Avery, and Cullen and Brown have recorded differences between strains of human and of bovine origin in relation to the final hydrogen ion concentration in dextrose broth. The hydrogen ion determination was carried out with our series of cultures in the hope that some differences might be detected. The beef infusion broth was made up with 1 per cent. dextrose, and had a reaction of pH. 7.4 after sterilization. It was inoculated with 0.2 cc. of eighteen hour broth cultures. The colorimetric method was used and readings were made after forty-eight hours incubation. None of the human cultures gave as low a pH reading as most of the bovine controls. The group (W. 168) already mentioned as distinctive with regard to colony morphology and hemolysis, was characterized by giving the lowest pH value among the strains studied.

The number of groups possible among the streptococci on the basis of hemolysis and fermentative reactions, the relatively low case incidence of hemolytic streptococci in the inflammatory conditions and the fact that when present, hemolytic streptococci represent only a very small fraction of the flora, even in the early acute stages, indicates without immunological study that a dominant variety of hemolytic streptococcus was not present among the cases investigated.

Agglutination Tests

In the serological investigation of the cultures, the type strains of Dochez, Avery and Lancefield were used to prepare serums for preliminary study. This was done by inoculating sheep with increasing doses of killed cocci followed by injections of live cocci. Antiserums were also prepared by immunizing rabbits with selected cultures of our own series. All injections were given intravenously.

The technic for direct agglutination is given in the original article.

Some cultures when grown in broth were clear or nearly clear due to spontaneous agglutination. In some instances these cultures on thorough shaking, followed by a short period of sedimentation gave a suspension which could be used. With other self agglutinating strains occasional transplants grew without spontaneous agglutination and these could be utilized. However, with quite a number of strains the degree of spontaneous agglutination was so marked and constant that they could not be used at all for agglutination tests. In general these differences as to spontaneous agglutination occurred irregularly without regard to any determinable factors. Many expedients have been tried to prepare uniformly suitable suspensions but without success. The tabulated results, therefore, are the optimum results and give no indication of the repeated attempts and the difficulties encountered.

The results of the direct agglutinations with the serums prepared with the Dochez, Avery and Lancefield strains are given in tables 1 to 8 of the original article. The primary interest is evidently with the piogenes and infrequens groups to which the serum strains belong. In the pyogenes group there are many instances of moderate or marked agglutination by the serums. With some strains this was evident with two or more of the serums. With other strains the action was only marked with one of the serums. Serum 3 agglutinated more strains than any of the other serums.

Absorption Tests

The next step in the study was the absorption of the serums with the strains agglutinated and also with a certain number of the strains which gave such a marked degree of spontaneous agglutination that no determination of their serological relationship could be made by direct agglutination.

The results of the absorption of serums, D. A. and L. numbers 23, 3, 84, 32 and 273, are given in tables 10 to 14 of the original article. It will be noted that in spite of considerable and often high cross-agglutination, only a very few absorption identities were encountered. The strains of case 122, I (influenza) absorbed the specific agglutinins of serum D. A. L. 32. Strain E (pleurisy) absorbed the agglutinins of serum D. A. L. 23.

Because of the peculiar colony characteristics of the strains from case 77, an anti-serum was prepared for this strain, using rabbits. This serum on direct agglutination reacted with all the strains of case 77 and with some of the other strains which fall fermentatively into the pyogenes group.

The absorption results show that all the strains from case 77 are alike. The other strains acted upon by this serum have not the capacity of absorbing the specific agglutinins. One strain, case 72, I, 3 (the only one which showed the same peculiar fermentative reaction as 77) was also used for absorption, although no direct agglutination reaction could be carried out because of spontaneous agglutination. This strain absorbed the agglutinins from serum 77. We have no data on the colony morphology. If the colony had been characteristic at the time of isolation, this point was overlooked. When the peculiar fermentative reactions of the strain were observed there was nothing characteristic about its colonies but by this time the colonies of serum strain 77 had lost their original peculiarities. The reactions of serum 77 with strains having colony characteristics similar to serum strain 77 but falling in other than the pyogenes group are discussed later.

Strain 18, I (4) and the strains isolated from case 168 with the exception of 168 V 1, fell in the infrequens group and are given in table 2. Only slight reactions were observed with D. A. L. 60 serum. An absorption, carried out with strain 168, I, 1, which gave the highest reaction in this group, resulted negatively. A rabbit serum for strain 168, I, 1 was prepared because of the similarity of its colony morphology to that of strain 77. All of the strains of case 168, with one exception (strain 168, V, 1) are absorptively alike. The one exception also differed from the other strains in its fermentative characteristics.

Six cases yielded cultures which fell in the equi group fermentatively, in that they fermented salicin but failed to ferment lactose, mannite and raffinose. These strains can be divided into two subgroups. Strains from cases 112, 116 and 167 form one group. Strains from case W and strain 168 V, (1) differ from the first group in their hemolytic activity, and in the final hydrogen ion concentration in dextrose broth and in their peculiar colony morphology. Because of these peculiarities an antiserum was prepared (rabbit) for strain W, I, 3 of the second group. This serum agglutinated equally well all the "W" strains, but gave only a slight reaction with strain 168, V, 1 and none with the strains from cases 112, 116, and 167. The cultures of the equi group were not agglutinated by the serums made from strains of the pyogenes group.

The absorption of serum W, 1, 3 showed that the W strains were identical, but that strain 168, V, 1, was different from these. Absorption tests of serum, W, I, 3, with two strains 112 and 116 of the equi group were carried out with negative results. These strains served as negative controls. It is worthy of emphasis that the W strains from pleurisy, approach the bovine type of streptococcus in many of their characteristics.

The strains which fell into the three groups, *Anginosus*, *Salivarius* a and b, and *Mitis* consisted mainly of the alpha prime type. These groups are small and the majority of the cultures were not usable for agglutination tests, because of their extreme spontaneous agglutination. On account of this fact, and in the light of the failure to obtain similarities in the larger groups, the preparation of further serums did not seem indicated. The serums of other groups were tried with alpha prime strains but gave no reactions, which is of interest in comparison with the cross reactions where serum strain and strains tested belong in the same fermentative group.

Colony Peculiarities

The colony distinctiveness of two groups of cultures "like 77" and "like W," have been mentioned previously in this paper. In the summary of the strains with the colony morphology "like 77" it will be noted that the colony similarity is not paralleled by common fermentative characteristics. The direct agglutinations are given in the tables according to the fermentative groups in which the strains belong. The results of the absorption tests showed that none of the strains which, as far as observed, had this colony morphology were capable of absorbing specific agglutinins from serum 77. Strain 72, I, 3 included in table 1 on account of its cultural similarity to strain 77 but whose colony formation had not been studied at the time of isolation is seen to be absorptively identical with strain 77. As noted under the discussion of the fermentation tests, this strain was the only one encountered which was similar to the strains from case 77 in having peculiarly low avidity for lactose.

The other group having a colony peculiarity is represented by the W strains and strain 168, V, 1. These strains were alike and peculiarly distinctive in all of the cultural and biological characteristics studied. Immunologically the W strains (isolated from the same specimen) are identical but strain 168, V, 1 differs from these. The results obtained with these distinctive groups of streptococci, show very clearly the fallacy of drawing conclusions as to the identity of strains even where the colony morphology and also all the other observed biologically characteristics are alike. If then, in a series of cases of respiratory inflammations, there is found such a distinctive type, its presence is only suggestive as regards probable etiology. No conclusions can be drawn as to the identity of such strains until a serological investigation including agglutinin absorption has been made. Without such investigation, the emphasis placed by some investigators upon the frequent isolation from influenza cases of a streptococcus having distinctive cultural characteristics is wholly unjustified.

A further study of the strains isolated from the various cases noted did not seem indicated. As the organisms were studied the tendency was toward the separation of groups, the number of which increased in proportion to the number of characteristics studied, and the prospect of encountering a dominant type became less and less promising. Moreover, the possibility of further serological investigation was limited by the extreme spontaneous agglutination of some of the cultures. The relatively low incidence of streptococcus hemolyticus and the relatively small numbers present when they did occur in inflammations of the mucous membranes also gave little indication that this organism was a common factor in any appreciable number of the cases investigated. Not only is there no evidence of a distinctive strain among the cases as a whole but likewise no indication of a distinctive strain common to any one of the types of disease studied.

Conclusions

A study of the streptococci of the hemolytic group obtained from inflammations of the respiratory tract shows that these strains fall into many cultural groups and sub-groups. A serological study showed so few similarities that the probability of a dominant strain seemed remote. There was no correlation between the grouping and the type of disease. These observations and the relative infrequence of streptococci of the hemolytic group in inflammations of the respiratory tract seem to justify the conclusion that none of the strains isolated were of primary etiological importance.

For bibliography see the original article.

STUDIES ON ACUTE RESPIRATORY INFECTIONS[1]

Differences in the Character of the Hemolytic Action of Streptococci and the Relative Value of Various Methods of Demonstrating These Differences

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Lucy Mishulow

Differences in the hemolytic activity of streptococci when grown on blood agar were noted by Schottmuller in 1903. The blood-agar plate was generally employed to determine the hemolytic ability of streptococci until Lyall (1914) advised the use of the tube method as a means of differentiation. Later Smith and Brown, using the blood agar plate method, introduced a new nomenclature to indicate the reactions obtained. Brown, elaborated these findings and gave detailed descriptions of the characteristics to be observed and advocated a classification based upon such observations. He gave a very complete review of the whole subject.

The superiority of either the plate method or the tube method depends upon the answers to the following questions: (a) Does the plate method bring out all the differential characteristics not shown by the latter method, or is the reverse true? (b) Does each method bring out certain characteristics? (c) Is there with either method a greater correlation between the results elicited and any determinable differences in the substances producing hemolysis?

The observations in this paper are offered in answer to these questions.

The streptococcus cultures employed were forty-two strains from the series reported by Valentine and Mishulow.

	Strains
Naso-pharynx, inflamed or "normal"	27
Pleurisy, purulent	4
Mastoiditis, blood culture	2
Tonsilitis	1
Serological Types of Dochez, Avery and Lancefield (6)	6
Brown (7) bovine and human "Types"	2

The strains were kept on blood streaked agar slants. Fresh transfers were made before each experiment.

The method of demonstrating hemolysis and the conditions of observation were as follows:

Blood plates: Two loops of an eighteen to twenty hour broth culture were transferred to a tube of sterile broth which was shaken thoroughly and two loops of this dilution were inoculated into 1 cubic centimeter of citrated horse blood. Ten cubic centimeters of liquid veal-agar were added and the mixture then poured into a Petri dish. By this procedure 10 to 25 colonies were obtained and the well separated colonies could be studied in detail. The plates were observed after twenty-four and forty-eight hours incubation at 37°C. They were then placed in the refrigerator for forty-eight hours and again observed.

The results recorded in a table are those noted after forty-eight hours in the incubator. With the cultures studied, forty-eight hours of refrigeration did not bring out any additional differential points.

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[1] This study is part of an investigation on acute respiratory diseases carried on under a grant from the Influenza Commission Fund of the Metropolitan Life Insurance Company. This series of studies was carried out under the direction of William H. Park, Anna W. Williams and Charles Krumwiede.

Tube hemolysis. (Lyall method.) The red cells from fresh defibrinated horse-blood were washed three times in 0.85 per cent. saline, and a 5 per cent. suspension in saline was made. To 1 cc. of this suspension fo red blood cells was added 0.5 cc. of an eighteen to twenty hour broth culture (beef infusion, peptone 1 per cent., NaCl 0.5 per cent., reaction pH 7.8 to 7.9), and after the tubes were shaken the mixtures were placed in a water-bath at 37°C. for two hours and were again shaken every half hour to prevent sedimentation of the blood cells. After this incubation, all tubes in which hemolysis was not complete were rapidly centrifuged to facilitate observation of the degree of hemolysis.

Brown's modification of the tube method. An eighteen to twenty hour broth culture was diluted 1 to 10 and 1 to 20 with physiological salt solution. To 1 c.c. of each dilution was added 1 drop of sterile defibrinated horse-blood and the mixtures were incubated in a water bath at 37°C. for two hours. Readings were made in the same manner as by Lyall's tube method. The proportion of red blood cells in different samples of defibrinated horse-blood varied considerably. To obtain uniformity in the amount of red cells used in the successive tests, the defibrinated blood was centrifuged and serum was added or removed until the volume of cells was to the volume of serum as 1 is to 2. The same broth cultures incubated at 37°C. for eighteen to twenty hours were used in making the hemolytic tests by the three methods described.

In tabulating the character of the hemolysis on the blood plates Smith and Brown's and Brown's nomenclature is employed. The strains that gave clear colorless zones of hemolysis in forty-eight hours with microscopically complete destruction of red blood cells around each colony were designated *beta*. Those strains that produced hemolysis macroscopically similar to the above, but the colonies of which on microscopic examination were found surrounded by blood cells, were termed *alpha* prime. The strains which produced no definite hemolysis after forty-eight hours incubation, but definite although usually narrow zones of clearing after forty-eight hours refrigeration, were termed *alpha*. The last group usually showed green discolorations in and about the colonies.

The tabulation of the results with the plate method (see original article, Table I), shows that of the 42 strains 32 gave the *beta* type hemolysis, 6 the *alpha* prime, and 4 the *alpha* type.

The question was raised in a few instances as to whether a strain should be classified as a *beta* or an *alpha* prime type. Most of these doubtful strains were finally considered *alpha* prime types because cells, although few in number and partly disintegrated, were still discernable about the colonies.

Two strains, 168 II(1) and 82 (1), gave wide clearcut zones of hemolysis, without any sign of red cells about the colonies. In the outer zone, however, there were visible on microscopic examination a few partly disintegrated cells. These strains were classified as *beta* types.

Five strains (168V (1), 10X, 11X, 12X and 13X) caused only slight hemolysis on the blood plate after twenty-four hours. After forty-eight hours there was a well defined zone of hemolysis and on microscopic examination no red cells were found about the colonies. Strain 168 V (1) was slower in its hemolytic activity than the other four strains mentioned. In retests this strain occassionally showed no hemolysis after twenty-four hours. All of the five strains because of the disappearance of the red cells about the colony were classed as *beta* types.

The results obtained with the tube methods, compared with each other and with the type of hemolysis on the blood agar plate (table 1), are summarized as follows:

Beta type 32; with Lyall method = 32 positive, 0 negative.
Alpha prime type 6; with Lyall method = 6 positive, 0 negative.
Alpha type 4; with Lyall method = 1 positive, 3 negative.
Beta type 32; with Brown method = 26 positive, 6 negative.
Alpha prime type 6; with Brown method = 4 positive, 2 negative.
Alpha type 4; with Brown method = 0 positive, 4 negative.

The six *beta* strains (10X, 11X, 12X, 13X, 168 V (1) and B cow 18) which failed to hemolyse by the Brown method gave variable amount of hemolysis by the Lyall method when retested. As noted above, five of these cultures (human origin) were also slower in producing hemolysis when grown on blood agar. These strains of human origin were characterized by further peculiarities. As is noted in table 1, the pH figure after growth in 1 per cent dextrose broth is the lowest for this group of strains. This figure pH 4.6 or 4.7 is usually indicative of bovine origin (Avery and Cullen; Brown. This is the figure given by strain B cow 18 (bovine origin).

These five human strains grew slowly and the amount of growth was never heavy. The failure to hemolyse regularly by the Lyall method may be due to the two factors; namely, the small amount of hemolysin present at any one time due to scant growth and the progressive deterioration of hemolysin in fluid media. The amount produced minus the amount deteriorated might be so small that either no reaction resulted or at most a slight reaction. When this amount of hemolysin was reduced by dilution (Brown's method) no reaction resulted. The hemolysis on the plate although slow was always well defined after forty-eight hours. The more regular end results obtained with the plate may be due to the traces of hemolysin which acted directly upon the cells as fast as formed, thus eliminating to a large extent a loss of effect due to deterioration. If this explanation is valid, the plate method reveals the effect of all or nearly all the hemolysin formed, whereas the tube method reveals only the effect of that which has not deteriorated.

It appears, therefore, that for the demonstration of hemolysis by streptococci the blood plate method is more reliable than the test-tube methods because a certain percentage of feebly growing streptococci may fail to show hemolysis in the test-tube. The plate method is also more valuable for preliminary classification because it brings out differences in hemolysis that the tube methods fail to show. Since the completion of this work Clawson has published similar conclusions with regard to the value of the blood plate. The Brown modification of the tube method has not been of value with the human strains reported in this paper. This method was used by Brown primarily for the differentiation of streptococci of bovine origin.

The failure of the tube method to differentiate between the *beta* and *alpha* types, which were differentiable by the plate method, raised the question as to whether the mechanism of hemolysis might not be different with these two varieties. If an essential difference could be demonstrated, this would be added evidence of the value of the plate method as a means of preliminary differentiation.

The following experiments were designed primarily to determine whetner hemolytic action might be due to an extracellar substance in the one case and an intracellular substance in the other.

An eighteen to twenty hour broth culture was shaken by hand to distribute the cocci evenly. One cc. of washed 5 per cent. horse-blood was added to 0.5 cc. of a sample of the broth culture (Lyall's method) and the mixture was incubated in a water bath at 37°C. for two hours, as a control. The remainder of the culture was rapidly centrifuged and the sedimented bacteria were suspended in distilled water for extraction. This suspension was made slightly more concentrated than the original broth culture and was divided into two parts. One sample (C) was placed in the ice-box and the other was shaken in a mechanical shaker for two and a half hours. A portion of the shaken sample (A) was tested immediately after shaking and the remainder (B) after further extraction in the icebox overnight. To obtain the extracts, all of the suspensions were centrifuged and to the clear extract was added 1/10 its volume of 9.0 per cent. sodium chloride to make it isotonic. The sedimented bacteria from each of the three extracts were resuspended in physiological saline and were also tested separately in portions A′, B′ C′. In each instance 1 cc. of a 5 per cent. suspension of washed red blood cells (horse) was added to 0.5 cc. of the test substance (extract or suspension of cocci after extraction) to determine the hemolytic activity.

The results are given in table 2 (see original article) and show that the extracts (refrigerator) of the *alpha* types had hemolytic action which was as strong as the original broth culture, or stronger. Extraction by shaking tended to destroy the hemolysin. Culture D.A.L. No. 32 (*beta* type), however, showed strong hemolysis immediately after shaking, which decreased after refrigeration. This result as well as that of extraction by refrigeration without shaking indicates that the hemolysin of this strain is more labile with regard to time than that of the *alpha* types.

The hemolytic activity possessed by the suspensions of the extracted cocci was relatively the same as with the original broth culture. The question whether this hemolysis was due to preformed substances or to the products of growth during the test remains unanswered.

No special study was made of methemoglobin production but as far as observed there was a sharp difference depending on whether extracts or suspensions of cocci were used; the former gave negative and the latter positive results when tested before refrigeration.

The question as to the persistence of the hemolytic activity of broth cultures of the various types was next considered. Broth cultures were made and the whole culture as well as the clear broth obtained by centrifugation were tested for hemolytic activity after incubation of the cultures for one, two, three, four and six days respectively, using Lyall's tube method (table 3 of the original article).

The *beta* and *alpha* types could not be differentiated by this method when the whole culture was used. With the supernatant fluid, however, there was a distinct difference. The *beta* types showed an average greater hemolytic activity after twenty-four hours incubation, which disappeared on further incubation. On the other hand the *alpha* types showed an increased hemolytic activity on longer incubation (see table 3). This seems to indicate that the hemolytic action of the *beta* type is essentially due to an extracellular product, labile in character; whereas that of the *alpha* types is due to the direct action of the cocci or of the products of their autolysis. The methemoglobin reactions were irregular, but it is noteworthy that they were consistently weak with the clarified broth.

The resistance to heat of the substances in cultures which cause hemolysis and the methemoglobin production was also investigated (Lyall tube method). The results of these experiments are given in table 4 (see original article).

There was a marked difference in the results obtained with the *beta* and the *alpha* types in regard to this point. With both *alpha* types there was a disappearance of hemolytic activity after heating at 55°C. for five minutes, while the *beta* hemolysin, resisted heating for five to ten minutes. These results apparently correlate and strengthen the deduction drawn from the previous experiments. Again, the indications are that the cultures of the *beta* type contain a preformed and least partially free hemolysin which resists heating for five to ten minutes. The hemolytic activity of the *alpha* types is apparently due to direct action of the cocci, or of substances liberated by autolysis. The heating for five minutes had probably killed a large number of cocci or inhibited their activity. As previous experiments indicate, the autolytic products had probably not developed in appreciable amounts during the eighteen hours incubation. The result of both these factors was an absence of hemolysis.

The results obtained in these studies probably explain to some extent the conflicting opinions of previous workers as to whether or not the hemolytic activity of streptococci is due primarily to an extracellular substance. The varying results obtained by different workers may depend on the types of streptococci studied and the methods used for investigation. De Kruif in a review of work of this nature has shown very clearly that with his cultures hemolysin contained in the clear broth reached a maximum concentration and then rather rapidly disappeared. The whole culture, however, retained its hemolytic capacity for a much longer time than the clear broth. These results correspond with the results of the filtration experiments of Meader and Robinson. They found that when a broth culture was rapidly filtered there was considerable hemolysin in the filtrate; whereas with slow filtration the filtrate gave negative results.

The conclusion that the *alpha types* owe their hemolytic activity primarily to direct action of the cocci brings up the question as to the mechanism of this action. With both the *beta* and the *alpha* types there are other factors involved besides the mere liberation of the hemoglobin. This is plainly evident with the blood plate method, where hemolysis, is accompanied by the development of a clear transparent decolorized area. The action of bacteria upon the stroma and hemoglobin together with the mechanism of this action has been investigated by several workers. We have not considered this aspect of the problem but have limited our efforts to the demonstration of primary differences in hemolytic action that would correlate with the blood plate picture, which depends not only upon simple hemolysis but upon the activity of other factors as well.

Conclusions

The blood agar plate, incubated for forty-eight hours, is a more reliable method of determining the hemolytic action of streptococci than the tube method, because differences in hemolytic action can be observed with the blood agar plate which are not observable with the tube method.

The differences in hemolysis observed in the plate are correlated with primary differences in the mechanism of hemolysis. The hemolytic action of the *beta* type is primarily due to a hemolysin which is a soluble extracellar product. The hemolytic activity of the *alpha* types seems to be due to the direct action of the cocci or to products liberated by autolysis. The plate method which brings out these points, is, therefore, the more valid method on which to base a classification.

A modification of the tube method, in which only the clarified broth is used for the test, appears to be an improvement on the regular method. However, it may fail in some instances to reveal hemolytic action where the plate method would give positive results.

For bibliography see the original article.

STUDIES ON ACUTE RESPIRATORY INFECTIONS[1]

The Nature and Value of a So-Called Precipitin Reaction as Applied to The Serological Grouping of Streptococci

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CHARLES KRUMWIEDE AND EUGENIA VALENTINE

Barnes, in a study on the cultural and serological relationship of hemolytic streptococci reported recently that the serums of rabbits intensively immunized against the streptococcus will give precipitin reactions with streptococcus antigens even when such serums are diluted as high as 1-3200.

His method of eliciting what he terms a precipitin reaction, was as follows: The streptococci were grown in broth for forty-eight hours at 37°C. The broth cultures were centrifuged at high speed to sediment the cocci. In each test a constant amount (1 cc.) of clear undiluted supernatant broth antigen was added to an equal amount of the diluted antistreptococcus serum. These mixtures as well as controls with normal rabbit serum and with uninoculated broth were incubated at 37°C. for eight to ten hours, removed to the ice-box for two hours and then read. Sterility tests were made to eliminate the possibility of the precipitate being bacterial growth.

To us the results as reported by Barnes seemed most extraordinary. In our experience the most potent serums would when added to concentrated antigens, give precipitin reactions only in dilutions as high as 1:100 or 1:200 at most. Even the prolonged incubation period used by Barnes did not seem to us to explain his positive results with thousand-fold and greater dilutions of serum.

It seemed advisable, therefore, to investigate the reaction obtained by Barnes to determine whether his interpretation of its nature was correct. If the reaction were actually a precipitin reaction the relative specificity of the results would be open to question; in addition the applicability of the antibody-absorption method would be limited, because the absorption of precipitins gives, not infrequently, non-specific results. Even if Barnes' interpretation were not correct, the reaction might have an advantage over the regular agglutination method, by eliminating the all too frequently disturbing factor of spontaneous agglutination.

Our experiences with the method described by Barnes when controlled by the regular agglutination methods are not given here in full. The table in the original article sufficiently illustrates our findings. These results also suffice to show the nature of the reaction.

The striking point in the table is the difference in the reactions obtained with the method described by Barnes when parallel tests were incubated at 37°C. and at 55°C. respectively. At 55°C. there is seen a reduction of the degree of reaction within the limits to be expected with the precipitin reaction and which we actually obtained with culture filtrate antigens. If the reaction described by Barnes were a true precipitin reaction there should not be this difference at the two temperatures employed.

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[1] One of a series of studies carried out under the direction of William H. Park, Anna W. Williams and Charles Krumwiede. The previous studies in this series were reported in this Journal, 1921, vol. 6, no. 1. This investigation was made possible by a grant of money from the Metropolitan Life Insurance Influenza Fund for a part of the expense.

Numerous tubes in both series were used for bacteriological tests. The contents of each tube was added to a separate tube of blood agar and the mixtures poured into petri dishes. After incubation, abundant growth was obtained from all the samples of the 37°C. series but from none of the 55°C. series. Likewise, smear and hanging drop examinations of the sediment of centrifuged tubes of similar series incubated at 37°C., showed the presence of numerous streptococci. Where clumps were visible to the naked eye, clumps of streptococci were also seen in the hanging drop preparations. The constant evidences of growth even from the saline control in every series incubated at 37°C. and the absence of such growth in the 55°C. series, absolutely contradicts Barnes' claim that the precipitate is not due to bacterial growth.

After we had obtained the above results, two statements in Barnes' article seemed very significant. He found that the addition of cresol 0.3 per cent "seriously interfered with the precipitin reaction." Likewise, he found that the supernatant broth from a culture gave "better results" than an extract from killed cocci. How much reaction was obtained and in what dilution, is not stated in regard to these extracts.

There is only one possible conclusion as to the mechanism of the reaction described by Barnes. Although with the immune serums there is some true precipitin action in the lower dilutions, the essential phenomenon is that of growth and agglutination of the cocci.

As to the value of the reaction it is evident from a study of the table that with cultures showing little tendency to spontaneous agglutination, the results were about the same as those obtained with the ordinary agglutination method. With cultures showing a marked tendency to spontaneous agglutination, no better results were obtained with the miscalled precipitin method which for convenience we shall term the growth-agglutination method. To determine the degree of spontaneous agglutination a comparison, dilution by dilution, of the results obtained with the immune and with the normal serum must be made. In the lowest dilutions the serum present may limit spontaneous agglutination because in these dilutions the cocci are growing in a medium qualitatively like serum broth. In the highest dilutions the amount of growth decreases and consequently the evidences of spontaneous agglutination become less marked.

In a few instances given in the table, the results have been somewhat better with the growth agglutination method than with ordinary agglutination. Thus with serum 3 and strain 3 the contrast between normal and immune serum is more marked. With the same serum and strains 23 and 171, the degree of group agglutination is less marked.

These results indicate that the application of the method might be attempted if the possibility of obtaining results was sufficiently important to overbalance its drawbacks, namely—the frequent failures due to spontaneous agglutination, the time for centrifuging, the excessive care necessary to prevent contamination and the difficulty of reading the relatively less marked reactions obtained. If this method were employed, the question would be raised immediately as to the applicability of the absorption method to evaluate the reactions obtained.

It does not seem of value to tabulate our absorption tests. As was to be expected from the data given in table 1 the results obtained were practically the same as with the regular agglutinin absorption method. Where spontaneous agglutination interfered with one method it interfered with the other. Somewhat greater difficulty was encountered with the growth method, as in some instances a larger absorbing dose was necessary than was the case with the regular agglutinin absorption method. In carrying out these absorptions the mixtures of serum and absorbing cocci were incubated at 55°C. to prevent growth of the absorbing strain in the subsequent tests. This method was satisfactory as was shown by the parallel tests with the regular agglutination method.

Conclusion

The method described by Barnes is not, as he concluded, a precipitin reaction. The reaction is due to the growth and agglutination of the cocci in the mixtures of broth and diluted serum. The results obtained by this method are essentially the same as those obtained by the ordinary agglutination method.

The method is of no especial value as regards the elimination of the factor of spontaneous agglutination which renders so difficult the serological investigation of streptococci.

For bibliography see the original article.

A PECULIAR FAILURE OF HEMOLYTIC ACTION IN A STRAIN OF HEMOLYTIC STREPTOCOCCUS

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Eugenia Valentine

While titrating by the tube method the hemolytic capacity of a series of streptococcus cultures of the *beta* hemolytic type, a peculiar absence of hemolysis was observed with one culture.

With the strain W 12, 0.5 cc. of an 18-hour broth culture failed to give hemolysis when mixed with 1 cc. of 5 per cent. washed red cell (horse), while 0.4 cc. of the culture plus 0.1 cc. of sterile broth resulted in almost complete hemolysis of the red cells.

Another strain having the same characteristics as W 12, but differing from it agglutinatively, also showed hemolysis in all the tubes.

The failure of the whole culture of strain W 12 to lake red cells until the addition of sterile broth, is of interest from the practical standpoint, for it may be a source of error in testing the hemolytic activity by the tube method usually employed. From the theoretical standpoint it seemed of interest to explain, if possible, the mechanism of this phenomenon.

In most of the further experiments, 0.5 cc. and 0.2 cc. of culture were used. The phenomenon was observed with marked constancy without regard to the kind of broth employed, that is, whether made of veal or of beef, the mode of sterilization, the period of storage and the variations of reaction within the limits suitable for growth.

It was found, however, that 0.5 cc. of a broth culture of W 12, which had been incubated for only six hours, caused hemolysis, without the addition of fresh broth, but that the same culture incubated for fifteen or eighteen hours did not. The failure of hemolysis by the 18-hour culture was always prevented by the addition of 0.1 cc. of sterile broth, and the addition of as little as 0.03 cc. of broth resulted in the development of some hemolysis.

When the clarified supernatant broth of an 18-hour culture of W 12 was used in place of sterile broth, no hemolysis took place. Heating this supernatant broth to 56°C. and 100°C. for fifteen minutes gave no different results. The supernatant broth from an 18-hour culture when added to a 6-hour culture did not prevent hemolysis. Adding peptone water to 18-hour broth cultures was as effectual in renewing hemolytic activity as the addition of sterile broth. Sterile horse serum, however, did not serve so well. Acid production was evidently not a factor, for neutralization of the small amount of acid produced did not result in hemolysis.

The outstanding points were therefore: the active hemolysis after six hours' incubation, the absence of hemolysis after long incubation, the renewal of hemolytic action by the addition of broth or peptone water, that is, a fresh food supply. This raised the question: Was the failure of hemolysis due to the cessation of growth and was the resumption of hemolytic action, after the addition of fresh broth, due to renewed multiplication?

This point was investigated by making poured plates. After 2 hours' incubation of the tube to which no fresh broth had been added, plate counts showed at most a slight increase in the number of cocci. The tube, however, to which fresh broth had been added, showed after two hours' incubation an increase of 30 to 60 per cent. in the number of colonies developing in the plates.

SUMMARY

It would seem that the failure of hemolysis of the strain of hemolytic streptococcus studied was due to cessation of growth which was renewed by the addition of a small amount of fresh broth. The reason, however, why this culture should present this peculiarity remains obscure.

For bibliography see the original article.

THE LOSS OF HEMOLYTIC CAPACITY BY A FRACTION OF A CULTURE OF A HEMOLYTIC STREPTOCOCCUS WITHOUT CHANGE IN AGGLUTINATION CHARACTERISTICS.

Published in The Journal of Experimental Medicine, 1922, Vol. XXXVI, pp. 157-162

By Eugenia Valentine and Charles Krumwiede

The partial or complete loss by the hemolytic streptococcus of its ability to hemolyze red blood cells has been recorded. In the studies on variations of streptococci as regards hemolysis and other characteristics, no complete and adequately controlled observations on the agglutination, agglutinin-binding, and agglutinogenic characteristics have been made, as far as we know, to determine whether variation in function has been associated with change in the antigenic constituents of the cocci. We are reporting, therefore, comparative observations on a strain of hemolytic streptococcus which has developed a non-hemolytic fraction.

The strain under consideration is one of the Dochez, Avery, and Lancefield type strains, No. 32, which had been isolated from the lung at autopsy (bronchopneumonia following measles).

Described according to the recommendations of Smith and Brown and Brown, the hemolytic fraction has all the characteristics of the *beta* types with a 4 to 5 mm. transparent zone of hemolysis. The green fraction is an *alpha* type with no visible clearing except for the late development of a very narrow green translucent zone, which microscopically shows scarcely any diminution in the number of red cells. Immediately surrounding the colony there is no reduction of the number of cells. The surface colonies of the green type fraction are moist, green-zoned, and resemble closely a typical pneumococcus colony.

The hemolytic properties of the two fractions were also tested with the blood tube method (Lyall). With the *beta* type, hemolysis was prompt; 0.3 cc. of a 15-hour broth culture caused complete hemolysis, and as little as 0.05 cc. caused partial hemoylsis. The green fraction showed no hemolysis with 0.5 cc.; the color of the red cells was changed to a deep magneta, but not to the typical brown shown by the pneumococcus used as a control.

Morphologically and culturally the two fractions show no differences, other than those noted above.

Agglutination and Agglutinin Absorption Tests.

Agglutination tests were set up with the serum of a sheep immunized against the original Strain 32. Fortunately all fishings of the hemolytic and green fractions grew with a diffuse cloud in beef broth so there was no difficulty in obtaining satisfactory agglutination results. The results of the test with the sheep serum showed that both fractions were agglutinated equally well, and to the titer of the serum strain, No. 32. Likewise the absorption of this serum by either fraction removed completely the agglutinins for both fractions and for the serum strain, No. 32.

For more conclusive evidence rabbits were immunized with a representative of each of the fractions. At each injection control plates were made to check the character of the injected cocci. The serums obtained were used for agglutination and agglutinin absorption tests.

Direct agglutination and agglutinin absorption tests, with the anti-serums for both the hemolytic and green fractions, were also carried out on the original Strain 32, and on the other fishings, H.2, H.3, and G.2, G.3 isolated at the time the cleavage was noted. The reactions of these strains were identical with those of the serum strains H.1 and G.1 given in Table I of the original article.

The ability of either fraction to absorb the agglutinins from the anti-serum of the other fraction shows that the loss of hemolytic power has not been accompanied by any change in the agglutinogenic complex of the cocci.

Virulence Tests.

The virulence of the two strains was tested by mouse inoculation with 18 hour broth cultures. With the hemolytic variety of 0.5 cc. killed uniformly, 0.25 cc. irregularly. The green type was less virulent, 1 to 1.5 cc. causing death, but only irregularly.

Attempts were made to increase the virulence of the green variant by mouse passage in the hope that the hemolytic capacity might return with the increase in virulence. After fourteen consecutive passages the virulence was raised so that $\frac{1}{60}$ cc. caused death. This increased virulence was not associated with the development of hemolytic colonies. The mouse passage caused no marked fall in the agglutinability and no change in the absorptive capacity of the culture. A further attempt to restore the hemolytic capacity to the green variant was made by growing it in blood broth, but twenty-five successive transfers in this medium have resulted in no change. The hemolytic fraction has also bred true after twenty-five blood broth generations.

Replating of the original Strain 32 showed the persistence of the two varieties throughout August, 1921. At that time fresh isolations of the two fractions were made and found to be identical culturally and serologically with the previous isolations. Since then, two platings of the original strain have failed to show any green colonies although a large number of plates were made.

Tests with Other Strains.

Twenty other strains of hemolytic streptococcus, the majority from pathological conditions, which had been under cultivation for from 2 to 8 years, were tested in order to determine whether or not any loss of hemolysis had occurred. Frequent platings of these strains have shown no change in the hemolytic capacity, as all colonies give well marked zones. The observations are in contrast with those of Clawson who found that out of 116 strains of hemolytic streptococcus under cultivation for 1 to 4 years, 54 showed one or more green colonies when plated in blood agar. He tested the constancy of substrains obtained from six cultures. His substrains showed little tendency to breed true, the green substrains in all instances showing reversions to the hemolytic type.

Conclusions.

A culture of hemolytic streptococcus developed a non-hemolytic fraction which as determined by its agglutinogenic, agglutination, and agglutinin absorption characters was identical with the hemolytic fraction, the only variation being one of function, or physiological action. This functional variation has proved permanent, as long as observed, for both fractions have continued to breed true.

This study is offered as additional evidence in favor of the hypothesis that functional changes among bacteria are, at most, only very infrequently associated with changes in the antigenic matrix of bacteria.

OBSERVATIONS ON THE CROSS PROTECTIVE POWER OF ANTI-PNEUMOCOCCUS MONOVALENT SERA, TYPES I, II AND III[1]

With a Note on the Cross Protection Afforded by So-called "Antibody Solution"

Published in The Journal of Immunology, 1924, Vol. IX, pp. 505-513

Betty S. Kolchin and Louis Gross

In 1922 Cecil and Larsen published a paper dealing with their observations on the results of treating pneumococcus pneumonia with Huntoon's Types I, II and III polyvalent antibody solution[2] where they brought out among other things the suggestion that the statistical results indicated that the mortality rate from group IV pneumococcus pneumonia was lowered by such treatment. Similar observations were reported by Conner in the same year.

This result was due either to an accidental fluctuation in the mortality rate of the treated and control cases or to genuine antibody protection against Type IV organisms by an antipneumococcus serum made from the three fixed pneumococcus Types I, II and III. This question of possible cross protection was considered by the Influenza Commission of the Metropolitan Life Insurance Society of great interest and practical importance. This investigation was undertaken by the writers at the suggestion of the Commission.

If it were true that considerable cross protection against group IV pneumococcus strains could be obtained by combined Type I-II-III antibodies, this would represent an encouraging step in the direction of serum treatment of the pneumonias due to this heterogeneous group.

Our first step was to try out cross protection in mice against group IV strains. In an attempt to confirm the clinical findings referred to above, we employed at first Huntoon's antibody solution.

We accordingly obtained fifty-one strains believed to belong to group IV which had been isolated from pneumonia cases in several large hospitals.

These strains were subjected by us to the agglutination test and all but three were confirmed as belonging to group IV.

Two of the three mentioned agglutinated typically with serum Type I, and the third one seemed to be a mixture of Type I and Type IV. In the latter case, after passages through mice the Type I strain seemed to have outgrown the Type IV strain and agglutinated typically with Type I serum. This admixture was also confirmed by making numerous fishings from the original culture and by subsequently agglutinating them. These agglutinations established the presence of two mixed strains in the original culture, a Type I strain and Type IV strain. These findings suggested that about 5 per cent. of identifications by routine typing are erroneous.

This report deals with the 48 remaining strains. The virulence of these strains was determined by injecting intraperitoneally graded doses of an eighteen-hour blood broth culture into mice weighing 18.5 to 21 grams. The graded doses were made up to 0.5 cc. volume with broth, and the smalles doses as expressed in fractions of 1 cc. of the original culture which killed within five days, was taken as indicating the virulence of the strain. Bacterial counts of these doses were determined by plating measured amounts of the culture in blood agar pour plates.

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[1] This investigation was aided by a grant from the Influenza Commission of the Metropolitan Life Insurance Society.

[2] Huntoon's polyvalent antibody solution is prepared by agglutinating heavy emulsions of the types, I, II and III pneumococci in polyvalent antipneumococcus sera with subsequent centrifugalization of the organisms and dissociation of the antibodies in weak alkaline solution.

If the virulence was less than such as would kill a mouse within five days after an injection of 0.00001 cc. the strain was subjected to a process of raising its virulence. For this purpose, we passed the strain through mice and tested the virulence after approximately ten passages.

It might not be out of place to mention here a point in the technic of mouse to mouse transfer of the pneumococcus strain, which we found very helpful. After intraperitoneal injection the mouse was usually found dead the next day. The dead mouse was pinned to a board and its fur soaked in 5 per cent. carbolic acid. With sterile scissors the fur was snipped along the sides and across the lower abdomen of the mouse. Grasping the lower border of the flap of fur thus freed, by means of forceps, it was torn over the head. This exposed the abdominal and chest wall untouched. The ribs were then cut through with sterile scissors, and removed. Almost invariably an abundant pleural exudate rich in organisms and free from contamination was found. This was sucked up into a sterile syringe and injected into a fresh mouse. The advantage of this method over that of sucking up the peritoneal material at the site of the previous injection lies in the fact, that the pleural exudate possibly contains the more virulent organisms which apparently passed through the blood system.

Agglutinating sera were prepared against the original strains as well as against the corresponding strains after the passages through the mice. The object of these sera was to determine whether the final strain obtained after the last mouse passage was identical with the original, and if any strains of group IV were found against which protection could be afforded, whether there was any serological relationship among them.

Of the 48 group IV strains which we obtained, 26 were ultimately of such a virulence that a mouse weighing 18.5 to 21 grams was killed within five days with doses of 0.00001 cc. or less. Thirteen of these strains were originally of a sufficiently high virulence and 13 had to be raised. Eleven strains showed no appreciable increase in virulence after 10 to 15 passages through mice, but were nevertheless tested for protection by the antibody solution. Eleven strains were so low in original virulence that no effort was made to raise it. These were discarded.

In the protection tests with the Huntoon antibody solution, the usual procedure was adopted. One-half cubic centimeter doses containing graded amounts of an eighteen-hour blood broth culture of a strain from group IV penumococcus were mixed with 0.5 cc. of a 40 per cent. solution of the Huntoon polyvalent antibody[3] and injected into a series of mice. The mice averaged 18.5 to 21 grams in weight and death within five days after injection was considered as standard. In this way we were able to pick out from among the 37 tested strains, three against which protection up to 1000 fatal doses was obtained. Even though two subsequent repetitions failed to confirm this protection, the apparent discovery of these three strains led us to further work with several interesting observations. We shall therefore have

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[3] The dilutions of blood broth cultures as well as of the antibody solution were made up with plain broth.

TABLE 1

PROTECTION TESTS AGAINST CULTURES OF FIXED TYPES I, II and III PNEUMOCOCCI WITH HUNTOON'S POLYVALENT ANTIBODY SOLUTION

DOSES OF CULTURE IN FRACTIONS OF 1 CC.	TYPE I PNEUMOCOCCUS		TYPE II PNEUMOCOCCUS		TYPE III PNEUMOCOCCUS	
	Huntoon's solution	Virulence control	Huntoon's solution	Virulence control	Huntoon's solution	Virulence control
1.0	Dead		Dead			
0.5	Dead		Dead			
0.1	Dead		Dead			
0.01	Living		Dead			
0.001	Living		Dead			
0.000,1	Living		Living		Dead	
0.000,01	Living		Living		Dead	
0.000,001	Living		Living		Dead	
0.000,000,1	Living	Dead	Living		Dead	Dead
0.000,000,01	Living	Dead	Living	Dead	Dead	Dead
0.000,000,001	Living	Dead	Living	Dead	Not done	Not done
Protection obtained against	10,000,000 doses		100,000 doses		No protection	

occasion to refer to these strains again. For the sake of brevity we shall refer to these as the "three strains."

The identity of the "three strains" with the corresponding original ones was checked up by means of agglutination and cross agglutination tests.

Under the belief that these "three strains" were genuinely protected and before we had obtained the results from the repeated protection tests, we

TABLE 2

PROTECTION TESTS WITH TYPE I HORSE SERUM AGAINST CULTURES OF FIXED TYPES I, II AND III

Anti-pneumococcus horse serum Type I

DOSES OF CULTURES IN FRACTIONS OF 1 CC.	PNEUMOCOCCUS TYPE I	VIRULENCE CONTROL	PNEUMOCOCCUS TYPE II	VIRULENCE CONTROL	PNEUMOCOCCUS TYPE III	VIRULENCE CONTROL
0.5	Living		Dead		Dead	
0.1	Dead		Dead		Dead	
0.01	Living		Dead		Dead	
0.001	Living		Dead		Dead	
0.000,1	Living	Dead	Dead	Dead	Dead	Dead
0.000,01	Living	Dead	Dead	Dead	Living	Dead
0.000,001	Living	Dead	Dead	Dead	Living	Dead
0.000,000,1	Living	Sick	Living	Dead	Living	Dead
0.000,000,01	Living	Dead	Living	Dead	Living	Sick
0.000,000,001	Living	Living	Living	Living	Living	Dead
Protection obtained against	1,000,000 doses		10 doses		10,000 doses	

decided to determine which specific ingredient of the Huntoon antibody solution was effective. We may say at the outset that the particular lot of the Huntoon solution tried out against the fixed Types I, II and III as a preliminary test, showed its best protection against Type I pneumococcus, less protection against Type II and no protection against Type III (table 1). These tests as well as those subsequently to be described were carried out in duplicate. It seemed quite clear, therefore, that the Type III antibodies were not responsible for the cross protection which the Huntoon solution seemingly afforded against the "three strains."

We desired at this point to investigate the protective specificity of monovalent anti-pneumococcus horse sera of the three fixed types. Unfortunately we were able to obtain only Type I horse serum where the history of the horse made it certain that it had never received injections with others than Type I pneumococcus. This serum tried out against the three fixed Types I, II and III showed definite but slight cross protection which was confirmed when repeated (table 2).

In order to investigate the cross protective power of Type II and III sera as well as to rule out all doubts as to the specificity of the antigen used to obtain these sera, we determined to prepare our own monovalent protective sera in rabbits.

As is seen in table 3, the sera obtained from rabbits also showed slight but distinct cross protection, and this despite the fact that they showed no cross agglutination with the other fixed type strains. It is to be observed that the cross protection obtained showed some reciprocal relation between Types I and III. Another interesting point brought out in this table, is that the cross protective potency varied with the different rabbits. This is all the more interesting when we consider that each rabbit was injected with washed and killed organisms obtained from the same beef infusion broth culture. Moreover, numerous fishings from the cultures failed to show by agglutination any admixture of the strains. We may say too, that on the whole the protective specificity of rabbit sera seemed to us to be greater than that of the horse sera.

TABLE 3

CROSS PROTECTION BY RABBIT MONOVALENT SERA

The figures indicate the number of fatal doses against which protection was obtained

	SERUM NUMBER	TYPE I PNEUMOCOCCUS	TYPE II PNEUMOCOCCUS	TYPE III PNEUMOCOCCUS
Type I sera.......	26	1,000,000,000	10,000	0
	28	10,000,000	0 to 10	1.000
Type II sera......	29	0	100.000,000	*
	30	0	10,000,000	100
	31	0 to 10	10,000,000	0 to 10
	35	0	1,000,000,000	0 to 10
Type III sera......	33	10,000	0	1,000,000
	38	100	0 to 10	100,000

*Not done, short of serum.

Finally, rabbit protective sera were tried out against the "three group IV strains." The results of these tests are shown in table 4. It is seen that of the three strains, two could not be protected against by these sera. In strain

11, however, cross protection was afforded. Marked protection was obtained from Type II serum and slight protection from Types I and III sera. Of the Type III sera, one afforded some protection, the other showed none.

TABLE 4

PROTECTION AFFORDED BY RABBIT MONOVALENT PROTECTIVE SERA AND FELTON'S SERUM AGAINST THE "THREE STRAINS"

The figures indicate the number of fatal doses against which protection was obtained

GROUP IV STRAINS	HUNTOON'S SOLUTION	TYPE I SERA		TYPE II SERA	TYPE III SERA		FELTON SERUM
		No. 25	No. 28	No. 31	No. 33	No. 52	
No. 9	0 to 10	0	0	0 to 10	0	0	0
No. 11	0 to 10	1,000	1,000	1,000,000	1,000	0	10,000
No. 22	0 to 10	0	0	0	0 to 10	0	0 to 10

TABLE 5

PROTECTION TESTS AGAINST CULTURES OF FIXED TYPES I, II AND III PNEUMOCOCCI WITH FELTON'S REFINED SERUM

DOSES OF CULTURES IN FRACTIONS OF 1 CC.	TYPE I PNEUMOCOCCUS		TYPE II PNEUMOCOCCUS		TYPE III PNEUMOCOCCUS	
	Felton's serum	Virulence control	Felton's serum	Virulence control	Felton's serum	Virulence control
1.0	Dead				Dead	
0.5	Living				Dead	
0.1	Living				Dead	
0.01	Living				Dead	
0.001	Living				Dead	
0.000,1	Living				Living	
0.000.01	Living				Living	
0.000.00.1	Living		Dead	Dead	Living	
0.000.000,1	Living	Dead	Dead	Dead	Living	Dead
0.000,000.01	Living	Dead	Living	Living	Living	Dead
0.000.000.001	Living	Living	Living	Living	Living	Living
Protection obtained against..	100,000,000 doses		No protection		10,000 doses	

A preparation of Felton's refined anti-pneumococcus serum which is prepared from Type I horse serum, was tested for its cross protective power against the three fixed Types I, II, and III pneumococci. The results are seen in table 5. This serum, as is seen in table 4, also afforded some protection against strain 11 but none against strains 9 and 22.

Discussion

The chief point of interest which arises from this work is the cross protection between types which is shown by horse as well as rabbit sera. In the latter, cross protection is found only in Types I and III sera. An interesting point, which was continually brought to our attention, was the more or less constant reciprocal relation between Types I and III. It appears from our tables that in so far as the production of protective antibodies is concerned, Types I and III pneumococci bear a stronger antigenic relation to one another than do either of these with Type II or the group IV strains tested by us.

The next point brought out, which though perhaps of less importance, was nevertheless the original question which we desired to answer, is that none of the 37 group IV strains which we studied, could be protected against by the use of Huntoon's polyvalent antibody solution. This sample of the solution moreover, contains no demonstrable protective antibodies against Type III pneumococcus.

The fact that the several monovalent rabbit sera afforded some protection to one of the "three group IV strains" despite the inability of Huntoon's solution to afford protection, suggests that the considerably greater potency of the rabbit sera was responsible for this. Nevertheless, no protection was afforded by these rabbit sera to either of the remaining two of the "three strains." This suggests that only occasional members of group IV are amenable to protection by the several monovalent sera. It is quite conceivable that had we carried out protection experiments with the rabbit monovalent sera against more of the group IV strains, some might have been found to be amenable to protection by large amounts of antibody.

The classification of sera by their agglutinin content, while very valuable, gives no accurate indication of their protective titer and certainly no idea of their cross protective power. We have tried to point out the importance of the latter and we hope that this short contribution may stimulate interest in this direction.

Further observations on the cross protective power of sera should be of great practical importance.

Conclusions

1. A sample of Type I monovalent anti-pneumococcus horse serum examined by us showed definite cross protection against the Type III strain.
2. Monovalent anti-pneumococcus rabbit sera also showed cross protection. The protective power against the homologus strains was on the whole greater than that of horse sera.
3. The cross protective titer of these sera varied with the rabbits from which they were obtained.
4. Cross protection while clearly present between Types I and III sera, was far below the protection titer against the homologous strain. It is probably too slight to have therapeutic effect.
5. Despite this cross protection, all the rabbit sera studied by us were specific in their agglutinin content.
6. Protection tests with rabbit monovalent sera were made against three group IV strains. In one of these strains protection was present to the extent of from 1000 (Types I and III) to 1,000,000 fatal doses (Type II serum), depending on type of serum employed and to a lesser extent upon the rabbit, from which the serum was obtained.
7. The importance of determining the cross protection power of monovalent sera has been discussed.

It is a real pleasure to record our indebtedness to Dr. William H. Park, Director of these laboratories, for his continued help and interest in this investigation.

For bibliography see the original article.

THE DISTRIBUTION OF THE IMMUNE BODIES OCCURRING IN TYPES I, II AND III ANTIPNEUMOCOCCUS SERUM

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Edwin J. Banzhaf

Avery states that the immune bodies occurring in Types I and II are completely precipitated by 38 to 42 per cent. saturation with ammonium sulphate, and that they were incompletely precipitated by (a) ammonium sulphate in less than 38 per cent. saturation, (b) saturation with sodium chloride, (c) dilution and saturation with carbon dioxide and (d) removal of crystalloids by dialysis. He states that the most practical purification appears to be precipitation by 38 to 42 per cent. saturation with ammonium sulphate. The higher saturation, *i.e.*, the 42 per cent., corresponds to about 47.6 cc. of saturated ammonium sulphate solution.

Felton finds that a 10 times dilution with distilled water containing 4 per cent. N/1 phosphoric acid per volume of antiserum will, with Type I .completely precipitate the immune bodies. He has been less successful with some of the antisera of Types II and III, especially those of low protective value. We have corroborated Felton's findings with Type I. Practically a complete precipitation of the immune bodies occurs when diluted with distilled water containing 4 per cent. N/1 phosphoric acid per volume of antiserum. Felton's same technique on Types II and III has given us varying results.

Using ammonium sulphate as a precipitating agent, we corroborated Avery's work in finding that the immune bodies were completely precipitated by half saturation with saturated ammonium sulphate solution. We also found that the methods of separation of the so-called euglobulin and pseudoglobulin by ammonium sulphate and sodium chloride showed immune bodies in both glubulins. We found, however, that that portion of so-called euglobulin which is precipitated with 30 per cent. saturated ammonium sulphate solution, saturated sodium chloride or 12½ per cent. dried sodium sulphate, contained after dialysis only 8 to 10 per cent. of the total immune bodies. This holds true for all three types.

Using Felton's technique for diluting these dializates, the white precipitate that is obtained contains practically all the immune bodies from these first fraction precipitates or so-called euglobulins.

The filtrate containing the globulins soluble in 30 per cent. saturated ammonium sulphate solution, and precipitated by adding saturated ammonium sulphate solution to half saturation contains after dialysis about 90 per cent. of the immune bodies.

The saturated sodium chloride soluble globulins, precipitated by the addition of only 25 per cent. saturated ammonium sulphate solution, contains after dialysis about 90 per cent. of the immune bodies. The filtrate containing the globulins soluble in 12½ per cent. dried sodium sulphate, and precipitated by adding dried sodium sulphate up to 18½ per cent. at 36°C., also contains, after dailysis, about 90 per cent. of the immune bodies.

Using Felton's technique for diluting these dializates, the white precipitate that is obtained contains practically all the immune bodies from these second fraction precipitates. This holds true for all three types. In all instances where Felton's white precipitate is obtained in the three types of pneumococcus antisera, the immune bodies are found in his white precipitate.

The albumins have no immune bodies, and no white precipitate appears when diluted with distilled water. Heating the antisera to 58°C. for two hours destroys about 60 per cent. of the immune bodies in all three types.

Heating to 56°C. for one hour does not impair its protection value.

All protection tests were made by Miss W. Carey Noble, to whom I am greatly indebted.

For bibliography see the original article.

PRODUCTION OF STAPHYLOCOCCUS ANTITOXIN IN HORSES[1]

Published in The Journal of Immunology, 1927, Vol. XIII, pp. 25-29

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It has been shown by one of us that most strains of *Staphylococcus aureus* from pathogenic sources produce a poison which in 0.1 cc. amounts causes marked necrosis when injected into the skin of rabbits. At the time of writing these papers we were not aware that von Lingelsheim as early as 1900 described experiments in which he inoculated subcutaneously 8 to 10 cc. of sterile filtrates of broth cultures of *Staphylococcus aureus* and obtained marked inflammatory changes and sometimes necrosis. More recently, Neisser and Wechsberg confirmed these findings with smaller injections (0.2 to 0.3 cc.) of toxic filtrates. These observers found as we did, that this necrotizing poison was destroyed when heated to 58° for one hour.

On further analysis we showed this poison to be an exotoxin, as the serum of rabbits immunized thereto neutralized it in multiple quantities. By the method of titration and calculation used, after 3 to 7 intracutaneous injections of increasing doses of toxin, rabbit serum contained 1250 to 5000 units of antitoxin.

With a view to the possible use of staphylococcus antitoxin in human infections of staphylococcus origin, we undertook the immunization of horses. This was thought advisable in order to get greater quantities of serum than could be obtained from rabbits, and because it was hoped that horses, as it has been shown in the production of the other antitoxins, would also prove to be a better staphylococcus antitoxin producer than the rabbit.

In some of the horses the first few injections were made by the intracutaneous route. After such injections there was usually considerable swelling at the site of injection and occasionally the formation of a scab, 2 by 3 cm. in diameter. The other injections were given subcutaneously. There was no apparent local reaction after the subcutaneous injection, but in those horses which were getting large injections of toxin at close intervals (see table) there was frequently a rise of temperature of 101° to 104°F.

Table 1 gives some of the data on the immunization of 7 horses with the staphylococcus toxin. It is seen that 5 of the 7 horses died during the course of immunization after six weeks to three months' treatment. These horses died with symptoms of weakness and prostration, and at autopsy showed nothing abnormal macroscopically. It is noted in table 1 that these horses had been given injections of toxin at short intervals, in most instances one to two days apart. Furthermore—not indicated in table 1—the injection doses were increased rapidly, so that after three weeks treatment they were getting 50 to 75 cc. of toxin. We believe that this strenuous treatment may account for the deaths, since the last 2 horses immunized (nos. 6 and 7), in

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[1] We are indebted to Dr. E. L. Cornman, Marietta, Pa. for the immunization of some of the horses and to Miss Anne Gunthur for the preparation of some of the toxin.

TABLE 1

HORSE NUMBER	PERIOD OF INJECTION	NUMBER OF INJECTIONS	AMOUNT OF FIRST INJECTION	INTERVALS BETWEEN INJECTIONS	TOTAL AMOUNT TOXIN INJECTED	LARGEST AMOUNT TOXIN INJECTED	DATE OF DEATH	APPROXIMATE NUMBER OF ANTITOXIN UNITS IN 1 CC. OF SERUM	DATES OF BLEEDING
			cc.		*cc.*	*cc.*			
1	March 2, 1925, through May 20, 1925	17	0.5	First 10 injections 3 or 4 days	1051	300	May 23, 1925	30,000	May 6, 1925
				Last 7 injections 7 to 10 days				30,000	May 19, 1925
2	May 18, 1925, through July 14, 1925	23	10	First 12 injections daily	1205	85	July 28, 1925	7,000	July 22, 1925
				Last 11 injections 4 to 10 days					
3	August 20, 1925, through September 28, 1925	15	10	1 to 5 days	670	85	October 5, 1925		
4	November 20, 1925, through December 31, 1925	15	10	1 to 5 days	610	60	January 2, 1926	7,000	December 28, 1925
5	January 9, 1926, through March 20, 1926	2	10	2 to 4 days except in 2. instances	995	100	March 31, 1926	15,000	February 26, 1926
				7 and 11 days				20,000	March 19, 1926
6	March 1, 1926	20	1	First 10 injections 3 and 4 days	2401	400		20,000	May 21, 1926
				Other injections 7 to 21 days				30,000	June 11, 1926
								20,000	July 1, 1926
								30,000	July 12, 1926
7	March 1, 1926	20	1	First 10 injections 3 and 4 days	2401	400		10,000	May 21, 1926
				Other injectons 7 to 21 days				10,000	June 11, 1926
								10,000	July 1, 1926
								25,000	July 12, 1926

which injections larger than 12 cc. were given at eight- to ten-day intervals, are in good condition at the present writing after five months treatment.

The amount of antitoxin produced by the horses is given in table 1 (column 10). It is seen that even in the horses which withstood the immunization for a short period, the amount of antitoxin produced was greater than that contained in our strongest rabbit serums and in several horses many times stronger.

In contrast with normal rabbit serum which seldom contains even slight amounts of staphylococcus antitoxin, normal horse serum usually contains a definite quantity of antitoxin. Of 5 normal horse serums titrated for staphylococcus antitoxin, 1 contained no antitoxin, 3 contained 50 units and one 100. A diphtheria antitoxin titrated contained 200 units.

The titration for antitoxin was carried out as in our previous work There are, however, a few points in carrying out the titrations which should be emphasized. They are as follows:

1. Only healthy rabbits should be used, as an animal not in good condition for any reason may not react even to a strong toxin.
2. A strong toxin, viz., one which produces a + reaction diluted 1:40 should always be used in any titration.
3. In every rabbit used for titrations of an antitoxin, controls of a mixture of antitoxin of known strength and the toxin, and toxin alone with equal amount of salt solution should be injected. In accurate determinations an added control of toxin diluted to the point where it will produce a plus reaction should be inoculated.
4. The antitoxin and toxin controls should always be the last injections made on the rabbit. This last point is important because if considerable excess of antitoxin over that necessary to neutralize the toxin is used in one or more mixtures injected in the rabbit, this excess of antitoxin in itself may wholly or partially neutralize the later inoculations and the titration must be rejected and set up again on another rabbit with higher dilutions of antitoxin.

For bibliography see the original article.

MENINGEAL CONDITIONS NOTED DURING THE EPIDEMIC OF INFLUENZA

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Josephine B. Neal

In reviewing the cases of meningeal involvement developing during the epidemic of influenza, three points attract attention:

1. Very few cases of meningitis have been due to the influenza bacillus. We have seen but two, and have examined the fluids of a third case. In previous years we have seen as many in an equal length of time. If this epidemic were due to the influenza bacillus, it seems strange that the organism did not more frequently invade the meninges.

2. During October and the early part of November, we saw a larger number of cases of epidemic meningitis than is usual at that season, and the number dropped off subsequently. The great majority of these cases gave a history of an attack, clinically influenza, immediately preceding the development of the meningitis. In some instances, the meningitis appeared as the influenza was subsiding, in other instances after convalescence was established. The cases themselves were not unusual in any way. Some were mild and some severe—about as the cases usually run. Cultures of the spinal fluids were made on blood agar as well as on glucose ascitic agar to see whether hemoglobinophilic bacilli were present with the meningococci, but there was no evidence of a mixed infection. Whether the resistance of the individual was lowered locally in the mucous membrane of the nasopharynx and generally by the attack of influenza, thus making him fall more readily a victim to meningitis, or whether the meningococcus attained a heightened virulence through its association with the causative agent of the influenza epidemic, is a question entirely open to discussion.

3. In about twenty cases we have seen patients, usually during convalescence from attacks clinically influenza, in which meningeal symptoms have developed. There were present headache, some stiffness of the neck, Kernig's sign, and extreme apathy and drowsiness—in many ways the picture of an early tuberculous meningitis. Lumbar puncture has revealed a clear fluid under increased pressure. This fluid has shown an increase from slight to marked, in the number of cells, with a preponderance of mononuclears, a well marked increase in the albumin and globulin, a normal reduction of Fehling's solution, and no organisms either by smear or culture. In most instances a Wasserman test has been performed, and in all cases have been negative. Guinea-pigs inoculated with a number of fluids suffered no ill effects. Such a spinal fluid in no way suggests the invasion of the meninges by a pyogenic micro-organism. The mononuclear cells and the normal reduction of Fehling's solution are practically never present in a pyogenic meningitis. This in addition to the absence of organisms by smear and culture is evidence against a meningitis.

This spinal fluid reaction is quite different from that usually found in meningism occurring in pneumonia, gastro-intestinal conditions in children and acute infections. In these conditions, while the meningeal symptoms are often more pronounced than in the cases above described, the spinal fluid, with rare exceptions, is normal in the chemical and cytologic constituents. The exceptions referred to occur mainly after severe and prolonged convulsions, in the convulsive type of whooping cough and in cases in which the puncture has been done shortly before death, the circulation having been depressed for some time. In these instances, the changes in the cytology and chemistry are, as a rule, less than have been found in the influenzal cases. The spinal fluid changes found in the cases described are similar to those in cases of poliomyelitis and poliencephalitis. From the spinal fluid examinations and the clinical picture it seems possible that we are dealing with an encephalitis secondary to the influenzal attack. With one or two exceptions, the patients have improved after the lumbar puncture and made a good recovery.

In the original article several case histories are given.

INFLUENZAL MENINGITIS

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Josephine B. Neal

Meningitis due to the Pfeiffer bacillus ranks fourth in our experience among the types of purulent meningitis.

Table 1 shows the incidence of the different forms of purulent meningitis among the cases of meningeal involvement that have been studied by the Meningitis Division during the past 10 years.

TABLE I

CASES OF PURULENT MENINGITIS

Epidemic Cerebrospinal Meningitis	541
Pneumonic Meningitis	61
Streptococcic Meningitis	49
Influenzal Meningitis	32
Staphylococcic Meningitis	9
Total	692

The cases of influenzal meningitis that have been reported and collected by various workers are given in a table in the original paper.

The cases reported by Simon, Wollstein, Torrey and Stone represent reports collected from the literature in addition to their own cases. So far as I have been able to determine there are no duplications. The cases credited to Koplik, Dunn, and the Meningitis Division were seen by the workers in question.

Dr. Wollstein made a careful study of the published reports and from these, in addition to her own investigations, concluded that in majority of fatal cases a general blood infection occurs. She also pointed out that the development of influenzal meningitis is frequently preceded by otitis, rhinitis, clinical "influenza," or other involvement of the respiratory tract.

In our series of cases the diagnosis was made by finding in the spinal fluid the gram negative hemoglobinophilic bacillus described by Pfeiffer. In smears from the spinal fluid these bacilli show a high degree of pleomorphism, a fact observed by several other workers. An interesting point was brought out by Dr. Povitzky, of the Research Laboratory who found that 4 out of 7 strains from cases of influenzal meningitis belonged by agglutination to the same type. On the other hand, the strains isolated from the respiratory tract during the epidemic of influenza, so-called, showed only a slight tendency to fall into groups when studied by Miss Valentine and Miss Cooper.

It is, of course, only by the bacteriological study of the spinal fluid that the diagnosis of influenzal meningitis can be made during life, as there is nothing in the history, the clinical picture of the case, or the other characteristics of the spinal fluid to distinguish it from other forms of purulent meningitis. Indeed, from a clinical standpoint, the disease is especially liable to escape diagnosis, since so large a proportion of the cases are found in infants in whom the diagnosis of meningitis is generally most difficult. It is evidently a disease of very young children. In our series of 32 cases, only 2 patients were more than 5 years old, one of these being 7 and one 18 years of age. Sixteen of our cases were in children one year old or less.

We were able to locate a probable primary focus in only 8 instances and, of these, 6 were cases of pneumonia, one clinical "influenza" and one a cold with cough. I am, however, unable to say whether or not these primary infections were due to the influenzal bacillus. It is certain that meningitis due to the meningococcus does not develope frequently during or immediately following pneumonia.

Lumbar puncture is all too often delayed because the physician feels he is dealing with a meningism or with a meningitis secondary to the pneumonia probably due to the pneumococcus or streptococcus.

As to the seasonal distribution of cases, a tendency is shown for the disease to develop in the fall and early winter. But it is particularly interesting to note that in the fall of 1918, when the epidemic of influenza was raging in New York City, we saw only one case of meningitis due to the Pfeiffer bacillus.

This has seemed to us good evidence against the Pfeiffer bacillus being the causative factor of this epidemic. If the organism had been present in such vast numbers and with such heightened virulence as to have caused such an epidemic, it seems impossible that there should not have been an increase in the number of cases of influenzal meningitis. This has been commented upon in an earlier paper, and Stone also called attention to the same point in his experience in Texas.

As stated in a previous paragraph, there is nothing characteristic in the symptomatology of influenzal meningitis. Two patients had even the hemorrhagic rash, which is so often considered diagnostic of epidemic meningitis. The course of the disease varies greatly, from two or three days to a month or more but is usually well over a week. A table showing in tabular form, certain points of interest in the cases we have seen is given in the original article.

A study of the 13 cases in which recovery took place, shows that the treatment was as follows: In nine instances lumbar punctures alone was used; in one instance electrargol was given intravenously daily for a week; in two instances anti-influenzal serum was used; in one, vaccine was given intraspinally.

An anti-influenzal serum prepared by Wollstein in 1911 produced favorable results in experimental influenzal meningitis in monkeys. This was the serum used in pateints who recovered as reported by Torrey. The preparation has not had a fair trial clinically as the disease is too rare to furnish an adequate number of cases and the serum is often administered late. Dunn reports that he has used it in 11 cases without a recovery, but that too few doses were given to furnish an adequate estimate of its value. We have used it in five cases. In three of these only one or two injections were given, in the other two the injections were repeated about ten times in one instance and fifteen times in the other. These last two patients showed temporary improvement clinically; the organisms decreased greatly in number and became largely intracellular; death, however, finally resulted. The serum certainly should be used when available.

A brief description of one case that recovered may be of interest. No. 28 in our series.

Phyllis McA., 2½ years of age, had been well except for a mild cold and cough four to six weeks earlier. She had not recently been in contact, as far as we know, with any sick person. She became ill, suddenly, August 21st, 1920, with headache, vomiting and fever ranging from 99° to 102°. Two lumbar punctures were done, August 22nd and 26th. The spinal fluids were sent to us for examination and the second one showed the Pfeiffer bacillus. On August 28th the child was seen by Dr. Jackson of the Meningitis Division. Twenty-five cubic centimeters of turbid fluid was withdrawn and 500 million of a stock influenza vaccine was injected intraspinally.

The child's mother objected to lumbar punctures especially in view of the fact that we could hold out little hope for recovery, and it was not possible to persuade her to permit regular treatment.

On August 31st another puncture was made, 1000 million of vaccine being given mixed with antimeningitis serum, as the smear from the previous fluid had showed coccoid forms which suggested that the meningococcus might also be present.

Further study showed that this fluid as well as the previous ones contained the Pfeiffer bacillus in pure culture. At this point the case showed some improvement, and no further treatment was permitted until September 9th, when the child became suddenly worse with a rise of temperature and vomiting. A puncture was made and influenza vaccine was again administered. This was repeated about every other day, the case showing considerable improvement. An autogenous vaccine was used as soon as it could be prepared. On September 18th the fluid for the first time gave a negative culture, the previous eight fluids having been positive. At this puncture in addition to the vaccine, anti-influenzal serum, (prepared by the Research Laboratory, but not available until this time) was given. Therefore, in this particular case at least, the serum cannot be credited with the disappearance of the organism and the ultimate recovery of the case.

The administration of combined serum and vaccine was repeated on the 20th and 22nd of September. On September 24th and October 2nd, lumbar puncture was done for the relief of pressure, no injection being made. The child showed steady improvement in general condition. Deafness which had developed early in the illness and had seemed to clear up, returned about the middle of September. It did not seem to be complete and was apparently showing a little improvement when the child went to the country the middle of October. Her general condition at that time was excellent.

In several cases of meningitis not due to the meningococcus, we have used a vaccine intraspinally with the hope that its gradual absorption into the blood stream would bring about whatever beneficial effects result from vaccine given intravenously without the degree of shock usually following that method of administration. We had the opportunity of making several injections in this way in two cases each of streptococcic and staphylococcic meningitis, with recovery in one case of staphylococcic meningitis and temporary improvement in the three other cases. The reaction following this treatment has never been very severe, and we have seen no harmful results, though in some cases we have worked up to as large a dose as five billion, diluted in about 10 cc. of normal saline. The vaccines used were either stock vaccines of the same variety as the organisms present, or autogenous vaccines when such could be prepared, as it was desired to get any specific effects that might be obtained in addition to the general protein reaction. Sampietro and others have recently recommended homologous vaccine in protein-shock treatment as it seems to give better results and to be less toxic.

The intravenous administration of vaccine has been used also in treating meningitis. Typhoid vaccine was used in this way in a case of epidemic meningitis on the Fourth Medical Division at Bellevue Hospital. This patient had developed a walling off of the spinal fluid, repeated lumbar punctures resulting in dry taps. The condition was getting worse and indeed seemed to be quite desperate. The intravenous administration of the vaccine was followed by considerable shock. The man improved temporarily but died about four months later.

A case of blocked epidemic meningitis in a baby, treated with intraventricular administration of serum followed by vaccine intravenously and recovery, has recently been reported by de Angelis.

Recoveries from purulent meningitis, due to organisms other than the meningococcus, are few. Most of these have occurred after lumbar puncture alone. In view of this fact, it cannot be asserted that intrsapinal vaccine treatment cured the case of staphylococcus and of influenzal meningitis above described nor that the intravenous administration of vaccine cured the case of blocked epidemic meningitis to which we referred. However, recovery did follow the use of vaccine in these cases.

In the absence of methods promising better results it seems worth while in addition to the use of a homologous serum where such is available, to try the intraspinal or intravenous administration of vaccine in suitable cases. This course would be indicated under the following conditions: in cases of meningitis not due to the meningococcus, in blocked forms of epidemic meningitis and in other cases of epidemic meningitis which do not yield readily to serum treatment.

For bibliography see the original article.

EXPERIENCE WITH MORE THAN ONE HUNDRED CASES OF EPIDEMIC ENCEPHALITIS IN CHILDREN

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Josephine B. Neal

The scope of this paper permits only the briefest reference to the extensive literature that has grown up during the last four years around epidemic encephalitis. The articles referring especially to the disease in children, however, have been comparatively few. Batten and Still, Netter, Comby, Heiman, and others have contributed to this phase of the subject, reporting, however, comparatively few cases. Indeed, earlier, it was thought that adults were much more subject to the disease than children. In fifty-four early cases studied by Netter, 77 per cent. of the patients were more than 15 years of age, and in London 86 per cent. of patients in early cases were more than 10 years of age. In an earlier article from the meningitis division, it was pointed out that perhaps a number of cases in children were overlooked, as approximately half our patients were 15 years of age or younger. Further epidemiologic study has confirmed this opinion. In a recent and very thorough study by Happ and Mason slightly more than half their eighty-one cases were in this age category. Up to the middle of May 1921, we had studied 274 cases in exactly half of which (137) the patients were 15 years of age or younger. The exact age distribution is shown in the accompanying table. One hundred and twelve, or 40 per cent., of the patients were 10 years of age or younger.

In contrast to this, in about 65 per cent. of cases of epidemic meningitis and about 90 per cent. of cases of poliomyelitis, the patients are under 10 years of age. As regards sex, about 50 per cent. more males than females are attacked by the disease, but this also holds true of epidemic meningitis and poliomyelitis. The seasonal distribution of our cases in New York City is indicated in the chart, and shows that the maximum

AGE DISTRIBUTION

Age	No of Cases
3 months	4
From 3 to 6 months	4
From 6 to 12 months	8
From 1 to 2 years	20
From 2 to 5 years	29
From 5 to 10 years	47
From 10 to 15 years	25
Total	137
From 15 to 20 years	15
From 20 to 30 years	56
From 30 to 40 years	33
From 40 to 50 years	24
Over 50 years	9
Total	274

number of cases occurs in the first quarter of the year. Our sporadic cases of epidemic meningitis during the last eleven years have shown the maximum number in April, May and June, while poliomyelitis is usually at its height during the summer months. While epidemic encephalitis is accepted as being an infectious disease, the percentage of those susceptible to it is certainly very low. In only one instance have we seen two cases in a family. The two patients became sick on the same day, and no outside source could be traced. The reports of instances of traceable contagion are very few. This difficulty in tracing infection is encountered also in epidemic meningitis and poliomyelitis, and may point to a high resistance to these diseases on the part of the majority of people. We have observed two or three points in connection with the study of our cases which may be of some interest. One patient while convalescing from encephalitis contracted epidemic meningitis, probably from a patient in an adjoining bed in the hospital. Instances of such direct contagion in hospitals, as we all know, are very rare. One patient gave a history of epidemic meningitis a year before. Two patients were mentally defective. Four had suffered from attacks that were probably poliomyelitis. One patient after a year of good health died from a second attack of encephalitis, so far as the diagnosis could be made in both instances.

No attempt has been made to divide our cases into clinical types because we are evidently dealing with a virus that may attack any part of the central nervous system or any combination of parts. This obviously gives rise to a great variety of types. Moreover, a given case in its development often passes through several phases representing different types. For these reasons, an attempt at classification has seemed undesirable.

Symptomatology

The symptoms in children do not differ greatly from those in adults. A rather larger percentage of children show a sudden onset, and the course of the disease is ordinarily shorter than in adults, although the mortality is approximately the same. The symptoms comprise both those of a generalized infection, such as fever, headache, vomiting, constipation and malaise, and those more directly referable to the central nervous system. The latter show an almost endless variety. Lethargy was so prominent a symptom in many of the cases that the term "lethargic" was earlier used in describing the disease. This, however, is by no means a constant symptom. Indeed, insomnia is an equally striking phenomenon in many cases, and in some patients both conditions may be present at different times. Convulsions are common, especially in younger children. In many cases delirium is present, occurring either for a considerable time or for short intervals, lasting not more than ten or fifteen minutes and being repeated frequently.

Ocular disturbances are of very frequent occurrence and are of wide variety. They may take the form of diplopia, blurring of vision, blindness, ptosis, either unilateral or bilateral, strabismus, nystagmus, and occasionally peculiar motions of the eyes. In one instance there occasionally took place convulsive twitching of the eyes which then snapped quickly to the left, and remained fixed for two or three minutes. These seizures occurred at frequent intervals during the height of the disease, but disappeared entirely as the patient recovered. In another case the eyeballs performed in succession a curious, nearly complete rotation with the pupils looking inward. While one eye was in motion the other eye remained quiet, but there was more or less twitching of the lips and of the face muscles on the same side.

Hippus has been observed in two or three cases in adults. I have not seen it in children.

Paralyses of wide distribution may occur. The cranial nerves are more often affected. Facial paralysis or paresis, either unilateral or bilateral, is often present and probably causes the masklike expression so often noted. In some instances the neck muscles have been affected; in one case this was unilateral. Inability or unwillingness to swallow may occur to such an extent as to make tube-feeding necessary. Disturbances of speech are not uncommon. The one I have observed most frequently is that in which the patient speaks in a slow, monotonous slurring voice, with considerable delay before answering questions. Some cases are extremely talkative. In a number of instances under my observation, patients have refused to speak for a varying length of time. Twitchings, either general or localized, are a very common phenomenon. They vary from a fine tremor to choreiform movements. Catatonia is not infrequent. The reflexes show all varieties of change. They may be increased, decreased, lost, equal or unequal. The Babinski sign and ankle clonus are not uncommon. Retention of incontinence of urine occasionally occurs. Constipation is often obstinate. Profuse sweating is fairly common. The more typical meningeal symptoms, such as stiffness of the neck and the Kernig sign, are not common, unless associated with some degree of general spasticity.

The course of the disease varies greatly, from two or three weeks to many months. The prolonged cases, lasting several months, are less common in children than in adults. The course of the disease is often marked by sudden rather transitory changes either for the better or the worse, and one must, therefore, be guarded in making a prognosis from a single inspection of the patient. A sharp and unexplained rise in temperature occasionally occurs. Pneumonia may develop during the course of the disease and is a dangerous complication. A rather common type of the disease in children is that in which there are none of these more striking phenomena. There is a history of a gradual onset, a varying degree of drowsiness, headache, vomiting, constipation, low, irregular temperature, and some changes in the reflexes. The diagnosis of early tuberculous meningitis is suspected, but after one or two lumbar punctures, in which the spinal fluid does not corroborate this diagnosis, the case clears up.

Laboratory Findings

The blood count in epidemic encephalitis varies from normal to one showing a moderate degree of leukocytosis, perhaps up to 15,000 or 20,000. Blood cultures are sterile. The urine is negative or shows a mild degree of nephritis common in acute infectious conditions. The examination of the spinal fluid throws more light on the subject than any other laboratory procedure. The spinal fluid shows practically the same picture as in poliomyelitis. It is clear and usually increased in amount. A web rarely forms. The cell count may sometimes be normal. It is usually moderately increased, ordinarily to no more than 100 or 150, though in one case it ran as high as 1500. The mononuclears usually predominate, but as in poliomyelitis there may occasionally occur a preponderance of polymorphonuclears. As in poliomyelitis, the fluid may in rare instances be slightly blood tinged, probably indicating a more than usually severe hemorrhagic process. The albumin and globulin are increased, usually slightly to moderately. The glucose, as measured by the qualitative reduction of Fehling's solution, is normal. Quantitative studies by various workers, among them Foster, have shown that it is sometimes slightly increased above normal. I do not feel that this knowledge is of great diagnostic value. Different workers vary in their estimation of the glucose in normal fluid. In quantitative work done in the meningitis division in 1916, is was found by Kahn that the glucose in poliomyelitis fluids frequently ran from 0.08 to 0.10 per cent., also an increase above normal.

The Wassermann reaction is negative. The colloidal gold curve is not characteristic. When the disease first made its appearance, many of the English writers and some in France and America reported normal spinal fluid findings, but most of the recent writers have reported findings similar to those described above. It will be seen from this description that these findings are not diagnostic of epidemic encephalitis, as similar findings may occur in poliomyelitis, in brain abscess, brain tumor, and except for the Wassermann test and colloidal gold test, in syphilitic conditions of the central nervous system, all being conditions in which the brain substance is primarily involved, with the meninges being secondarily affected. Such findings also occur early in tuberculous meningitis. They do show, however, that there is an inflammatory reaction in the central nervous system, and they are of value in ruling out other conditions in which meningism occurs with symptoms referable to the central nervous system but without an actual inflammation being present.

While much work has been done to determine the specific etiologic factor of epidemic encephalitis, the results obtained by different workers have been by no means in agreement. The work of Loewe, Strauss and Hirshfeld, has been in part duplicated by Levaditi and Harvier. Other workers have reported negative results in their attempts to corroborate this work. In view of these facts we must feel that the exact etiologic factor has not been fully established. Loewe, Strauss and Hirshfeld, and Levaditi and Harvier believe that they have established that the disease can be transmitted to rabbits and monkeys, and that it is due to a filterable virus in many respects similar to that considered to be the cause of poliomyelitis.

Differential Diagnosis

Since this disease is so protean in its clinical manifestations and since there are no laboratory findings that are absolutely specific, the diagnosis must be made largely by exclusion. In the case of children, tuberculous meningitis is the disease from which it must be most often differentiated, and this differentiation is by no means easy. The onset, while usually gradual in both, is more often abrupt in encephalitis, and in this type of case death may occur within seven to ten days, a shorter duration than tuberculous meningitis usually has. On the other hand, patients suffering from encephalitis sometimes die after a course of two or three months, a longer period than cases of tuberculous meningitis show. Diplopia is occasionally present in tuberculous meningitis, and transient paralyses especially of the cranial nerves are common. So also is loss of reflexes. Other neurologic manifestations that have been described in epidemic encephalitis are rare. Evidences of tuberculous lesions elsewhere, positive tuberculin reaction, tubercles in the choroid and so forth, are also of help in making the diagnosis of tuberculous meningitis. The examination of the spinal fluid in the last analysis must often be the final factor. While the spinal fluids may not be dissimilar early, as a rule even fairly early the spinal fluid in tuberculous meningitis will have a higher cell count and a greater increase in albumin and globulin than is the rule in encephalitis, even though the reduction of Fehling's solution be normal. As the disease progresses, the reduction of Fehling's solution in cases of tuberculous meningitis will practically always become diminished or absent, and it is possible in a large percentage of cases to demonstrate the tubercle bacilli in the smear. If this cannot be done, the result of animal inoculation must be awaited. Films form almost invariably in fluids from tuberculous meningitis, and only occasionally in fluids from epidemic encephalitis.

The diagnosis from poliomyelitis, especially the encephalitic form, is practically impossible except by neutralization tests of the blood serum. These tests are difficult to perform and are not always conclusive. Amoss has shown that the serum from recovering cases of encephalitis has no power in neutralizing poliomyelitis virus. Of course, the onset of poliomyelitis, even of the encephalitic type, is more frequently abrupt than is that of encephalitis. There is likely to be a higher temperature early, which soon subsides; there is more frequently diarrhea in poliomyelitis, while constipation is the rule in encephalitis. The pupils in poliomyelitis practically always react to light, while in encephalitis the reaction is often sluggish. Moreover, even in epidemics of poliomyelitis, the encephalitic form is rare. The age and season must also be considered. All these factors are helpful, although not final, in differentiating the two conditions. The spinal fluid findings are so similar that they are of little aid in making the diagnosis.

Brain tumor and abscess of the brain must also be differentiated; and these conditions have proved a stumbling-block to very able neurologists. Brain tumor is comparatively rare in children. It is usually afebrile, and a roentgen-ray examination should be of value. A choked disk is, of course, much more common in this condition than in encephalitis. The spinal fluid findings vary greatly in brain tumor, so that in this condition they will not be of so much diagnostic value. Abscess of the brain is rare and is usually accompanied by some demonstrable primary focus. In abscess of the brain, the blood culture is frequently positive.

Syphilitic conditions of the central nervous system must always be considered, and they may be ruled out with a fair degree of assurance by the result of the Wassermann test and the colloidal gold test of the spinal fluid. Cerebral hemorrhage, thrombosis or embolism are of less importance in children than in adults. Of course, a cerebral embolism with an endocarditis occasionally occurs in children, and must be differentiated from both poliomyelitis and encephalitis. Uremic conditions must also be considered, and must be ruled out by the urinary examination, and the test of the kidney function.

The diagnosis from various acute forms of purulent meningitis is usually easy clinically, and may be made absolutely by the examination of the spinal fluid.

Of course, the diagnosis must also be made from acute infections in children, in which convulsions develop, and sometimes stupor, such as typhoid, pneumonia, and severe gastro-enteritis; but these can usually be easily ruled out by finding the underlying cause and by various laboratory tests, as well as by a test of the spinal fluid, which, in these conditions, is usually normal.

REPORT OF CASES

Case 1.—F. A., a girl, aged 5 years, with no history of previous illness, had a gradual onset about October 1, 1920. The child was listless and apathetic, slept a great deal, vomited once; had no headache; the bowels were constipated, and there was loss of sphincter control of both bowel and bladder about October 12. When examined, October 20, she was apathetic, the temperature was normal, respirations were regular, and the pulse varied in rate from time to time; the pupils were equal, and reacted to light; there was no stiffness of the neck nor Kernig sign, the reflexes were present and equal, and there was no paralysis. Lumbar puncture gave about 35 cc. of clear fluid under pressure. This showed a slight increase in mononuclear cells, and also in albumen and globulin. The reduction of Fehling's solution was normal. Soon after this the child refused to speak. This lasted for about two weeks. Then speech returned, and the incontinence finally cleared up about the middle of November. At last reports she showed complete recovery physically, but was still nervous and irritable.

Case 2.—G. L., a girl, aged 5½ years, gave a history of poliomyelitis in 1916, with paralysis of right arm and leg. The paralysis of the leg had nearly cleared up but that of the arm remained. The onset of the disease occurred the latter part of January, 1921, with headache, vomiting, convulsions, and a temperature up to 103 F. When seen February 14, she was crying out constantly and apparently could not see. The temperature was still around 103 F., and 20 cc. of clear fluid was withdrawn. This fluid showed 460 cells—90 per cent. mononuclears, albumin and globulin + + +, and Fehling's solution normal. After the puncture, the temperature subsided. The child screamed almost constantly for several days, and then began crying real tears. She paid no attention to questions, indeed, did not speak at all. These symptoms lasted about ten days, then the condition improved. The child began to speak but was at first slow in remembering and in speaking. The sight returned. The condition was very satisfactory until March 13, when the child was taken for an automobile ride. In the afternoon, she had a convulsion and became unconscious, Cheyne-Stokes respirations developed, with feeble, rapid pulse, and marked cyanosis. Lumbar puncture was done in the afternoon, showing clear fluid, greatly increased pressure, 35 cells, albumin and globulin + +, and Fehling's solution normal. Next day the patient seemed practically well, but continued to have a slight rise of temperature for several weeks. She is now reported as entirely recovered.

The next case has been previously reported, but is included now on account of the sequelæ.

Case 3.—E. M., a girl, aged 14, gave a history of measles, and paralysis of the left arm and leg, probably poliomyelitis, which developed some years before and had practically cleared up. The present illness developed rather slowly, beginning about January 12, with apathy, tremors and subnormal temperature (96 F.) the chief symptoms. She had grown progressively worse, and when examined, January 16, had the appearance of being in a very serious condition. Her temperature was still 96 F., she was sweating profusely, catatonia had developed, and there were marked tremors. She was extremely apathetic and apparently in a semistuporous condition, but she answered questions correctly, though very slowly and in a monotonous voice. The neck was moderately stiff and there was a question as to the Kernig sign, since there was generally spasticity of the muscles. The knee jerks were equal and exaggerated. The pupils were equal and responded to light. There were no disturbances of vision. Lumbar puncture revealed clear fluid under increased pressure, a moderate increase in cells, 90 per cent mononuclear, albumin and globulin + +, and Fehling's solution normal. For about a week following, the temperature was slightly elevated; the neck was rigid and the patient was troubled with insomnia. Thereafter she slept for about seventeen hours, and then began to improve rapidly. She returned to school and seemed perfectly well until late in June, 1920. During the summer of 1920 she became very indifferent, disliked doing anything about the house, or taking care of her person, and was extraordinarily slow in accomplishing any task, even a very simple one. When seen in October, memory was good, but she spoke in a monotonous voice, and very slowly, and the face was expressionless except for frequent silly giggles. The muscles were spastic and reflexes exaggerated, but she was able to walk about, though very slowly and with a spastic gait. This condition became progressively worse, and when seen in May, 1921, she was unable to walk without being practically carried. She could not sit down or rise from a chair without a great deal of assistance; there was a constant tremor of the hands and feet; the muscles of the whole body were extremely spastic; she had difficulty in swallowing, and the voice was monotonous and slow; but memory was still good, and she seemed to have considerable insight into her condition. She still had a rather silly laugh, but it seemed to come from difficulty in muscle control rather than from lack of intelligence. She had obstinate constipation and there was frequency of micturition. Such a development after four or five months of apparent recovery is a striking commentary on the sequelæ which so often follow this disease. The final outcome of this case will be a matter of great interest.

SEQUELAE

That the effects of the disease may be prolonged for many months and that symptoms may develop after a period of apparent return to health has been pointed out by Abrahamson, Happ and Blackfan; Leahy and Sands; Happ and Mason and others. I have tried as far as possible to follow up our recovered cases at varying intervals.

On account of the great interest centering around the sequelæ, I have included those in adults as well as in children. They seem to develop more frequently in adults than in children. Up to the end of March, sixty-three cases, or 39.3 per cent. of the recovered cases, showed sequelæ for varying lengths of time after the subsidence of the acute symptoms. In these cases, twenty-one patients were less than 15 years old, and forty-two were adults. A part of this difference may be due to the greater ease in eliciting in adults certain conditions, such as headache, mild grades of mental deterioration, or lack of power to concentrate. It is, of course, too early to decide as to the permanence of the sequelæ. However, three out of ten recovered patients, in 1918 are still showing some results of the disease. And it is possible that some of the more recent cases that now seem to be complete recoveries may develop sequelæ in the next few months.

Like the symptoms, the sequelæ are also varied, and it is difficult to group them in any very satisfactory way. Moreover, one patient often shows several sequelæ.

In a general way they may be loosely classified as:
Weakness or paralysis: Adults, 8; children, 4.
Tremor, choreiform movements, spasm; Adults, 6; children, 3.
Pains in body or limbs: Adults, 2.

Headache: Adults, 8.
Dizziness: Adults, 3.
Nervousness: Adults, 5; children, 6.
Change in disposition: Adults, 1; children, 3.
Insomnia: Adults, 3; children, 2.
Drowsiness: Adults, 1; children, 3.
Speech defects: Adults, 6; children, 3.
Defect of Eyes: Adults, 9; children, 4.
Change in mentality: Adults, 14; children, 10.

The changes in mentality are of great interest. In children there are usually indications of mental deterioration—the child is demoted in school, when previously it had made good progress. In adults there is loss of memory and of ability to concentrate, in the milder cases, and sometimes mental depression. In one instance a young woman committed suicide during convalescence. One man was at a hospital for the insane for some months, but was finally paroled. A woman has been at such an institution for over a year.

It is interesting to compare these frequent, diverse and serious sequelæ with those of poliomyelitis and epidemic meningitis. In the former, the sequelæ are certainly sufficiently grave, but they are confined almost entirely to flaccid paralyses. In meningitis only a very small percentage of sequelæ occurs. The most serious is deafness, and this occurs in only 3 or 4 per cent of cases. Blindness, due practically always to a panophthalmitis, is still more rare. While a popular opinion exists that mental defects are often due to meningitis, a careful study of recovered cases of meningitis fails to corroborate this opinion.

Relapses after comparatively short periods of improvement occasionally occur. In one instance, already referred to, there was a second attack, at the expiration of a year of good health, which proved fatal.

MORTALITY

The mortality reported by various writers has varied greatly. In France it has been around 25 to 30 per cent. and in England as high as 50 per cent. In this country, Abrahamson reports a mortality of only 10 per cent., and Happ and Mason a mortality of only 7.4 per cent., while others have reported a mortality as high as 40 per cent. Smith gives a mortality of 29 per cent. In our series of cases, it has been about 28 per cent.

TREATMENT

The general treatment is of great importance and consists, in brief, in general hygienic measures, keeping the patient comfortable, quiet, and well nourished. Feeding by gavage may be necessary. There must be careful attention to the elimination, as constipation is often persistent and obstinate, and retention may occur. While numerous drugs and intraspinal injections have been used, the degree of success attending any of these measures has been small. Lumbar punctures for the relief of pressure have seemed of value to me and are recommended by many writers. The patient should be carefully watched during convalescence, and one should not be too hopeful of a complete cure until some months have passed.

For bibliography see the original article.

A STUDY OF FOUR HUNDRED AND FIFTY CASES OF EPIDEMIC ENCEPHALITIS

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Introduction. Since epidemic encephalitis was first recognized, an enormous literature has developed in continental Europe, England and the United States.

In this paper no effort will be made to review this literature, as our object is simply to present the outstanding features that have developed in the study of a rather large number of cases.

These cases were seen beginning in the fall of 1918 and during the following five years up to 1924. The 1924 cases, 81 in number, have not been included, as we wish to emphasize somewhat the after effects of encephalitis and it is still too early to report on the sequelæ of these cases.

In the fall of 1918 we first realized that we were beginning to see cases of a type that was new to us. Since 1910 the meningitis division has been studying cases of meningeal involvement, the different kinds of meningitis, poliomyelitis, meningism occurring in the various acute infections and other rarer conditions involving the central nervous system. This experience gave us a fairly good background on which to study this interesting disease.

At first we did not recognize that the cases were epidemic encephalitis. We were misled by the reports in the English literature, that the spinal fluid findings were negative, and also by the fact that the English reported that the cardinal symptoms were lethargy, asthenia and cranial nerve palsies. With rare exception, the spinal fluids in our cases have not been normal. While the triad of symptoms described by the English is very characteristic when present, many cases do not present all three and some not even one of these symptoms. As time has passed it has been recognized more and more that cases of epidemic encephalitis present a widely varied symptomatology. The term encephalitis lethargica seems to us very unfortunate, since it emphasizes a symptom which is frequently not present. It is very difficult to convince a patient or his family that he has encephalitis lethargicia when insomnia and restlessness are the outstanding symptoms.

The following, Tables I, II and III, show the distribution of cases by year, season, sex and age:

TABLE I
DISTRIBUTION BY YEARS AND SEASONS

Year.	Jan. Feb. Mar.	Apr. May. June.	July, Aug. Sept.	Oct., Nov., Dec.	Total.
1918	0	0	1	10	11
1919	26	4	9	29	68
1920	65	19	11	16	111
1921	49	25	25	9	108
1922	18	34	8	11	71
1923	45	17	14	6	82
Total	203	99	68	81	451

From the above table it will be seen that the greater number of cases occur in the first three months of the year and the smallest number in the summer months.

TABLE II.
DISTRIBUTION AS TO SEX

Year	Male	Female
1918	5	6
1919	52	16
1920	68	43
1921	60	48
1922	34	37
1923	45	37
Total	264	187

From the above table will be seen that approximately 59 per cent. are males and 41 per cent. females, or a little less than a ratio of 3 to 2. The preponderance of males over females is slightly less than in epidemic meningitis and poliomyelitis. In these diseases about 50 per cent. more males than females are attacked.

It will be seen from Table III that of the 451 cases 234 will more than fifteen years of age and 217 cases were less than fifteen years of age. This is a larger proportion of cases in childhood than is reported by most workers. But we see an unusually large number of cases in children because many physicians in the city are accustomed to call us to help them in the diagnosis of meningeal conditions in children.

TABLE III
AGE DISTRIBUTION

Age.	1918.	1919.	1920.	1921.	1922.	1923.	Total
Under 6 mos.	0	3	3	2	2	0	10
6 to 12 "	2	2	2	2	3	1	12
1 to 2 years	0	5	11	11	5	6	38
2 to 5 "	1	10	8	13	8	8	48
5 to 10 "	3	7	21	19	10	9	69
10 to 15 "	1	7	13	5	5	7	38
15 to 20 "	1	7	5	9	6	10	38
20 to 30 "	2	10	22	24	10	12	80
30 to 40 "	0	9	17	9	6	11	52
40 to 50 "	1	7	8	7	5	8	36
50 to 60 "	0	1	0	7	9	6	23
over 60 "	0	0	1	1	1	4	7
							451

For some time we have felt that cases in children were frequently overlooked. If we have made an unusually large percentage of mistakes in the diagnosis of epidemic encephalitis in young children we have made it consistently. Up to the middle of May, 1921, when 274 cases had been studied they were equally divided between patients more than fifteen years of age and those younger. Our findings are corroborated by the work of Happ and Mason, whose age distribution of cases is almost the same as ours.

In contrast to this, about 65 per cent. of cases of epidemic meningitis and about 90 per cent. of cases of poliomyelitis are less than ten years of age.

Symptomatology. While the chief symptoms are those referable to the central nervous system, there are also symptoms, such as fever, which is usually low and irregular, vomiting and malaise, which may be caused by a general toxemia.

The onset is usually gradual but in a certain percentage of cases it may be abrupt. It is more likely to be sudden in children than in adults. The symptoms referable to the central nervous system are of the greatest possible variety and vary widely in the same patient during the course of the disease, which is often greatly prolonged. This indicates that we are dealing with a virus which may involve any part or combination of parts of the central nervous system and which may progress from one part to another during the illness. This is in marked contrast to the action of the virus in poliomyelitis which attacks suddenly and has usually progressed to the maximum within five to seven days. In poliomyelitis the action of the virus is far more selective, and the greatest damage is done to the cells of the motor area, especially to the cells of the anterior horns of the spinal cord.

We have made no attempt to divide our cases into groups. The multiplicity of symptoms necessitates so large a number of groups that the classification becomes too unwieldly for the use of the general practitioner. Moreover, on account of the progress of the disease the same patient would often fall into any one of several groups, depending on the time at which he was seen.

So much has been written on the symptomatology of epidemic encephalitis that our discussion will be brief. We do wish to emphasize the fact that quite often the symptoms are mild and in other instances different from those originally considered pathognomonic. We feel that the diagnosis is missed in many instances because the physicians are looking for lethargy, asthenia and cranial nerve palsies. While extreme lethargy and somnolence are very characteristic when present, a fairly large group of cases suffer from restlessness and insomnia. In some patients both somnolence and insomnia are present at different stages of the disease.

Ocular disturbances of various kinds are frequent occurrence. Attacks of diplopia or blurring of vision are sometimes the earliest distinctive symptoms. These attacks may last for only a short time in the early stages or they may occur at intervals for a considerable period. Sometimes the diplopia or blurring is fairly continuous over most of the illness. Loss of vision, usually temporary, is occasionally seen. Other phenomena that are sometimes noted are: Ptosis, either unilateral or bilateral; strabismus, nystagmus and hippus. In one instance there occasionally took place convulsive twitchings of the eyes which then snapped quickly to the left, and remained fixed for two or three minutes. These seizures occurred at frequent intervals during the height of the diseases, but disappeared entirely as the patient recovered. In another case the eyeballs performed in succession a curious, nearly complete rotation, with the pupils looking inward. While one eye was in motion the other eye remained quiet, but there was more or less twitching of the lips and face muscles on the same side. While a large percentage of cases show a normal fundus, a slight haziness of the disc has been seen in a certain number of cases. Various degrees of optic neuritis with or without choking has been observed less often. Sometimes in late cases there is secondary optic atrophy.

In addition to the lethargy or excitability which are very common symptoms, and have already been referred to, delirium is not infrequently present. The delirium may last for a considerable time, or may occur at frequent intervals, lasting only for a few minutes. In other instances the patient is acutely maniacal for varying lengths of time. Catatonia is occasionally seen.

Sensory Disturbances. Headache is an almost constant symptom. Lancinating pains due to involvement of the posterior nerve roots are fairly common. These affect most commonly the arms and legs, but the thoracic muscles are sometimes involved. Numbness or tingling are less frequent symptoms. Occasionally there is loss of sensation, involving one or both legs and lower part of the trunk, resembling a hemitransverse or a tranverse myelitis.

Trophic Disturbances. These are rare but do occur during the early or later stages of the disease. Repeated ulcerations of the cornea were seen in one patient beginning during the comparative early stages and lasting for several months. In another case there was an extensive furunculosis late in the disease, and another case, also late, there were frequent attacks of stomatitis, thought to be trophic in origin. In one case the teeth decayed very badly after the attack of encephalitis and in another there was alopecia.

Motor Symptoms. One of the most striking of the motor symptoms which is found in a certain proportion of the cases is hypertonicity of the muscles. This is shown in the masklike expression of the face, in the general muscular rigidity and the slow movements. The rigidity of the neck due to this general hypertonicity must be sharply differentiated from the rigidity due to inflammation of the meninges. Muscular twitchings are a common phenomenon. They may be of several varieties. Myoclonic contractions may involve a single muscle or any group of muscles. Tetanic spasms are less common. Occasionally a persistent hiccough is seen. Tremors of various types frequently occur. They may be very fine or assume a choreiform or athetoid character. Convulsions are common in children. Paralysis or pareses are very frequent. Involvement of the oculomotor apparatus has already been referred to. Weakness or paralysis of the muscles of deglutition is comparatively rare. Facial palsies may be either of the central or peripheral type. Practically any form or combination of forms of extremity paralysis or paresis may occur, and this is usually of the spastic type, although not invariably so. The disturbances of speech are a little difficult to group. Some in which there is an obvious dysarthria are probably due to disturbed function of the motor mechanism. In other instances there is a great delay in answering questions. Speech may be hesitant, slow or indistinct.

The reflexes, superficial as well as deep, may be normal, diminished or exaggerated and may be equal or unequal. An Argyll-Robertson pupil is often seen, while loss of accommodation reflex is less common.

The Babinski and confirmatory signs are often present, and the Brudzinski sign is common. The Kernig sign is frequently present. Both patellar and ankle clonus are occasionally obtained.

Other symptoms may be noted, most of these evidences of a systemic infection. Fever is a very constant sign. It is usually low and irregular. Occasionally it is subnormal for a part of the illness. Vomiting is fairly common early in the disease. An obstinate constipation is quite characteristic. Jaundice is sometimes seen but is rare. Bladder disturbances are not infrequent. There may be either incontinence or retention. Hematuria has occurred in two or three instances. Disturbances of the respiratory rhythm occasionally occur. They may take the form of very rapid respiration of forced inspiration. The Biot type is fairly common. Cheyne-Stokes is rare except in the terminal phases. Profuse sweating is a rather characteristic sign and occurs not infrequently. Epistaxis has been noticed as an early sign but has been seen in a very few cases.

It may be well to say a few words about the symptomatology in children, as it is our impression that the diagnosis is often missed. In many cases the symptoms are milder than in adults; in others they are more atypical. Probably the most common form in children is that resembling an early tuberculous meningitis. In these cases the diagnosis can be made only by repeated examination of the spinal fluids. In a certain number of cases the children show symptoms quite unlike those we consider characteristic of epidemic encephalitis. The following is a case in point:

G. L., a girl, aged five and a half years, was seen, February 14, 1921. She had had poliomyelitis in 1916, with paralysis of the right arm and leg. The paralysis of the leg had nearly cleared up. The present illness had begun three weeks before with headache, vomiting, convulsion, delirium and irregular fever reaching 103°. No lumbar puncture had been done. On February 14 she was crying out constantly in a meaningless fashion and was apparently blind. A lumbar puncture was done, yielding 20 cc. of clear fluid with characteristic findings. The temperature began to subside after the puncture but the screaming persisted for several days and then she began weeping. This lasted for two or three days. Then the condition began to improve. The sight returned, the child began to speak rationally, but was slow in remembering and speaking. The improvement continued until March 13, when after an automobile ride she had a convulsion and became unconscious. Cheyne-Stokes respiration developed, the pulse was feeble and there was cyanosis. Another lumbar puncture was done that afternoon, yielding a clear fluid under increased pressure. This was followed by immediate improvement, and the next day she seemed as well as she had been before the convulsion. The improvement continued, although for several weeks she had a slight rise in temperature every second or third day. Three years later she was reported to be in excellent condition with no after effects.

Differential Diagnosis. The symptomatology of epidemic encephalitis is so varied that a complete diffential diagnosis would necessitate a discussion of a very large number of diseases of the central nervous system and many of the acute infections. We shall attempt to discuss the differentiation from only the most important of these.

Tuberculous meningitis is probably the disease from which epidemic encephalitis is most often to be differentiated. The diagnosis may be absolutely made by examination of the spinal fluid and therefore time will not be taken to discuss the different points of clinical diagnosis as they are often misleading. Diplopia, for example, occasionally occurs in tuberculous meningitis. The finding of tubercles in the choroid also makes the diagnosis of tuberculous meningitis certain. The differentiation from the other forms of meningitis is less difficult, but even here mistakes are sometimes made. In purulent meningitis diplopia or disturbances of vision sometimes occurs. A lumbar puncture, of course, immediately makes the diagnosis.

The differentiation from poliomyelitis is usually made, especially from the spinal form. From certain forms that have been described in the past as the encephalitic type of poliomyelitis, the diagnosis at present is a matter of personal opinion. Certain authorities consider that cases previously diagnosed as the encephalitic type of poliomyelitis were really cases of epidemic encephalitis, and it is possible that the classification of poliomyelitis will have to be revised.

The differential diagnosis from brain tumor or abscess of the brain is often made only after extensive radiographic studies or at autopsy. These conditions are so infrequent that the percentage of error in a large series of cases is exceedingly small.

Syphilis of the central nervous system in some cases can only be differentiated by laboratory aid, the Wassermann test.

Cerebral hemorrhage and thrombosis are often mistaken for epidemic encephalitis and *vice versa.* The differential diagnosis is often extremely difficult, especially from cerebral thrombosis. A hemorrhagic fluid makes the diagnosis of cerebral hemorrhage very probable, although blood-tinged fluids may be found in cases of encephalitis.

Cardionephritic conditions, especially in the pre-uremic stages, frequently resemble epidemic encephalitis. Studies of the blood chemistry and determination of renal function will prove the determining factors in establishing final diagnosis in this type of case.

Certain forms of mental disease, dementia precox, for instance, often present signs and symptoms similar to those of epidemic encephalitis. The case may have to be followed for some time before a definite diagnosis can be made.

Mention must also be made of encephalitis following acute infections, especially measles. The history of a recent acute infection will be the determining factor in making the diagnosis as the symptomatology and spinal fluid findings may be similar in the two conditions.

Laboratory Aids in Diagnosis. The blood examinations is not at all characteristic in epidemic encephalitis. The blood count usually shows a low leukocytosis from 10,000 to 15,000 but sometimes a little higher. The blood cultures are sterile. The analysis of the spinal fluid is the most diagnostic laboratory aid. The spinal fluid is usually clear and increased in amount; sometimes it is slightly hazy and it may at times be yellowish. There have been instances where we obtained a blood-tinged fluid. The cells are usually slightly or moderately increased, the cell count being ordinarily less than 100, with a preponderance of mononuclears. Occasionally there may be a larger increase in the number of cells up to 1000 or more. A preponderance of polymorphonuclears may at times occur. With but few exceptions we have found a slight or moderate increase in albumin and globulin content. Fehling's solution is promptly and well reduced, denoting a normal or even more than a normal amount of sugar in the spinal fluid. Of late we have used the quantitative method of determining the amount of sugar in the spinal fluid, and we have found the figures to range between 50 mg. and 100 mg. of sugar per 100 cc. Bacteriologically the fluid is sterile. These findings, while characteristic, are by no means pathognomonic, since similar findings are obtained in poliomyelitis, in some instances of syphilis of the central nervous system and early in tuberculous meningitis.

It may not be amiss to discuss in more detail the importance of the sugar content of the spinal fluid. Certain writers have stated that an increase in the sugar content is pathognomonic of epidemic encephalitis. While it is frequently found in epidemic encephalitis, it is also found occasionally in poliomyelitis, syphilis of the central nervous system and even in meningism. The determination of the spinal fluid sugar is of most value in differentiating tuberculous meningitis. In this disease, when well advanced, the spinal fluid sugar is usually, but not invariably, greatly diminished.

Mention should also be made of the Wassermann test which should always be done when there is any suspicion of syphilis. The colloidal-gold reaction is not so important, since luetic and paretic curves are fairly common findings in epidemic encephalitis.

In differentiating tuberculous meningitis from epidemic encephalitis, if the routine spinal-fluid examination is not definite, recourse must be had to guinea-pig inoculation.

In a few instances where the diagnosis was unquestionably epidemic encephalitis we have found a normal spinal fluid. Other writers have reported a fairly large proportion of normal spinal fluids. However, a study of the spinal fluid analysis in many of our leading hospitals has convinced us that such examinations are often incomplete.

Pathology. Epidemic encephalitis belongs to the class of inflammatory diseases in which also are included poliomyelitis, syphilitic lesions of the central nervous system and trypanosomiasis. While these different diseases have, broadly speaking, certain characteristics, the cases in a given class differ so widely that it is difficult, if not impossible, to accurately diagnose by a study of the pathology alone, the less typical cases.

The meninges are usually described as showing only slight changes—an increase in the cellular content, particularly in the neighborhood of the blood vessels of the pia-arachnoid. The cerebral cortex is generally normal, except for congestion of the vessel of the leptomeninges. In the brain substance the changes are most marked in the basal nuclei of the brain, the upper part of the pons and peduncles, the gray matter of the floor of the fourth ventricle and the aqueduct of Sylvius. The changes in the medulla and cord are often reported as less pronounced, though observers have noted the same changes occurring in the upper section of the cord. This was certainly observed in one of our cases, an adult, which came to necropsy. (Reported in the *International Clinics,* vol. 2, series 29.)

The lesions are generally described as consisting of four kinds:

1. Infiltration of the walls of the small vessels with lymphocytes and plasma cells.

2. Foci of interstitial and parenchymatous infiltration with round cells. In this reaction neuroglia cells may take part.

3. Lesions of the nerve cells—usually not so extensive as in poliomyelitis and with less neuronophagia. These lesions of the cells usually occur when the inflammatory process takes place in the gray matter, but they may develop in the absence of an inflammatory reaction. Such is the case with regard to the cells of Purkinje in the cerebellum where inflammatory changes are almost entirely absent.

Foci of perivascular hemorrhage. The vessel walls are usually not necrosed.

In connection with the statement that lesions of the cells may occur in regions where there is no evidence of inflammatory reaction, it is interesting to recall that Abramson, in a very excellent study of the pathology of poliomyelitis made at the Research Laboratory during the epidemic of 1916, brought out the same fact in regard to the lesions of poliomyelitis.

Treatment. While many methods of treating epidemic encephalitis have been tried, there has been such a lack of uniformity in results that it is safe to say that at present there is no specific treatment. The majority of observers have agreed that lumbar puncture is beneficial. Some cases seem to do better with repeated lumbar punctures; others are apparently unaffected. None of the cases we have observed have suffered ill effects from lumbar puncture. No definite rule as to the repetition of lumbar puncture can be followed. We have been guided by the clinical condition and the reaction to lumbar puncture, also by pressure symptoms and amount of fluid removed. In some cases the beneficial effects following lumbar punctures have been striking. The following case illustrates this:

N. P., a man, aged twenty-nine years, was seen, January 1, 1923. Past history and family history are unimportant. Present illness developed rather suddenly on December 25, with chills, headache and fever, 103.5°, but without vomiting. On January 1 the patient still had a temperature of 103°, stiffness of the neck, slight ptosis of the lids and double vision. Patellar reflexes were slightly exaggerated. There were signs of bronchitis in both lungs. The patient was quite nervous and very excitable. The headaches and excitability were gradually getting worse while the diplopia was diminishing. Physical examination was otherwise negative. Lumbar puncture was done and 40 cc. of clear spinal fluid under markedly increased pressure was obtained. On examination the spinal fluid showed a large increase in cells; albumin and globulin, + +; sugar was normal. The patient apparently derived so much relief from the puncture that one was done every other day, seven in all. On January 14, twenty days after the onset, the patient was apparently all right except for slight weakness. He made an uneventful recovery without any sequelæ, and is at work and entirely well today. This is apparently an instance where repeated lumbar punctures proved beneficial.

Except for lumbar puncture, the treatment is symptomatic. The symptoms are so varied that the length of this paper will not warrant a full discussion.

The mortality in our series of cases is given in Table IV.

Course of the Disease. When epidemic encephalitis first appeared it was considered a somewhat acute infection of the central nervous system, more or less like poliomyelitis. As time has gone on and cases have been studied further this opinion has been revised. Now we know that in many instances it assumes a very chronic course with periods of remissions and exacerbations. Sometimes relapses with acute symptoms occur after one or more years of apparently good health. It is not certain whether these are due to a lighting up of the old infection or to a new infection. One of our patients died of a second attack after being apparently well for a year. In other instances a year or more after the patient has apparently recovered, a slowly progressive condition, for example, a Parkinsonian syndrome develops. This is usually considered a sequela, but the opinion has been expressed that

TABLE IV.—MORTALITY.

Age.	1918.	1919.	1920.	1921.	1922.	1923.	Total	Mortality as to age, per cent.
Under 6 months	..	..	1	1	..	..	2	20.0
6 to 12 "	1	..	1	1	1	..	4	33.3
1 to 2 years	..	3	7	4	2	1	17	45.8
2 to 5 "	..	3	3	2	3	4	15	31.2
5 to 10 "	..	3	9	2	5	..	19	27.5
10 to 15 "	..	1	3	..	..	1	5	13.4
15 to 20 "	1	2	3	..	..	1	7	18.4
20 to 30 "	..	2	8	4	5	2	21	26.2
30 to 40 "	..	1	3	3	..	1	8	15.3
40 to 50 "	..	3	3	1	2	1	10	27.0
50 to 60 "	..	1	..	4	6	4	15	68.2
Over 60 "	..	..	1	..	..	3	4	57.1
Total deaths . .	2	19	42	22	24	18	127	
Total Number of cases	11	68	111	108	70	83	451	
Mortality	Per cent. 18.2	Per cent. 27.9	Per cent. 37.8	Per cent. 23.8	Per cent. 34.3	Per cent. 21.9	Average mortality	28.2

this is a secondary attack or a relapse. In other cases these chronic conditions and progressions follow the more acute stages without any marked improvement in the patient's condition. In general, the course of the disease is similar to that of syphilis of the central nervous system. It must not be forgotten, however, that a fairly large number of cases have remained well over a period of four or five years. The chronicity and variability of this disease is well brought out by the following case:

The patient, H. F., a female child, aged two years, was taken ill suddenly on August 18, 1923, with fever, 102°, and profuse sweating. The child was also gradually becoming progressively drowsy. The family and past history were negative. The physical examination on the fifth day of her illness showed the child to be acutely ill and apathetic. The temperature was 101°; pulse, 90; respiration, 20. There was definite rigidity of the neck. There was double internal strabismus. All the deep reflexes were normal, but the abdominals could barely be elicited. The Kernig, Brudzinski and Babinski signs were all negative. There was a definite weakness of the muscles of the back. A diagnosis of meningitic type of poliomyelitis was made at that time. Lumbar puncture was performed and 30 cc. of clear fluid under increased pressure was obtained. On examination the fluid showed a moderate increase in cells, 98 per cent. of which were mononuclears, a slight increase in albumin and globulin and a prompt and good reduction of Fehling's solution. On smear and culture no organisms were found.

October 2, 1923: There was a marked generalized rigidity, particularly of the neck. The strabismus had practically cleared up. The child also presented a typical picture of the Parkinsonian syndrome. There was also Parkinsonian tremor of the hands. It was now obvious that the child was suffering from encephalitis. Lumbar punctures performed at that time yielded 30 cc. of clear fluid under marked increased pressure.

October 17: Child still had Parkinsonian syndrome but to a lesser degree. The tremor was less marked and the child was able to walk a little. The neck was still rigid. Her general appearance was much brighter. On lumbar puncture 45 cc. of clear fluid were obtained.

June 12, 1924: The child was very much improved, but the Parkinsonian appearance was still quite obvious. There was neither rigidity of neck nor tremor of hands.

April 1, 1925: The child showed marked improvement. She was well nourished, bright and cheerful. The Parkinsonian features were disappearing, though her speech was still slow and the facies slightly masked. Her teeth were badly decayed. There was now a new complicating feature—a definite right hemiparesis of the lower motor neuron type as all her deep reflexes on that side were absent. She was unable to walk except when supported and guided. She also had slight inconstant tremor of her right hand.

This case illustrates a number of very interesting points. In the first place it shows how difficult it is sometimes to make a diagnosis of encephalitis in children. This would have been considered a case of poliomyelitis were it not for the Parkinsonian syndrome that developed two months after the onset of her illness. The chronicity of this disease is also very clearly brought out. The clinical picture has changed a great deal during the different periods of a two-year illness, indicating the involvement of different foci each time. The Parkinsonian syndrome, for instance, was a rather early complication, while later in the course of her illness a right hemiparesis developed as the Parkinsonian picture was clearing up.

We cannot, therefore, consider any of these cases as completely recovered or the sequelæ as permanent until many years have elapsed. It will, indeed, be very interesting and instructive to study a number of years from now the children who have had encephalitis.

Sequelae. It is difficult to obtain a very adequate idea of the sequelæ in epidemic encephalitis. Those who work in neurologic clinics are likely to be unduly impressed with the frequency of sequelæ, since patients with sequelæ go to clinics, while those with complete recovery do not. To obtain a true idea of the frequency of sequelæ it would be necessary to follow a large number of cases for a long time. In the first place, certain patients have symptoms lasting for a year or more, which eventually disappear. For instance, one patient was blind for a year but eventually recovered his sight. Two had change of disposition for about a year and one of them did poorly in school, but both eventually made complete recoveries. In several other instances there were sequelæ lasting several months which eventually disappeared. On the other hand, other patients are in apparently good health for some time, and then develop symptoms which may be considered sequelæ or recrudescences, apparently of a progressive character. Care should, of course, be taken to ascertain that the patient was free from abnormality before the illness. A rather large percentage of our cases had previously suffered from some defect or from some acute infection of the central nervous system before developing epidemic encephalitis. For instance, 2 were definitely mentally defective before the illness; 1 had Little's disease and had developed epilepsy; another had had epilepsy for several years; 1 had had chorea; 3 had had poliomyelitis; 1 had had epidemic meningitis; 1 died from epidemic meningitis while convalescing from epidemic encephalitis.

Of our 451 cases, 127 died. Of the 324 surviving, 36 for one reason or another could not be followed up, leaving 288 cases which were fairly adequately investigated. It was found that 78, or 27 per cent., showed definite sequelæ, while the remainder were carrying on their usual occupations, or in case of children were progressing in school satisfactorily, and to all intents and purposes showed no evidence of abnormality. Many patients showed several residual defects. Although the number of cases was nearly equally divided between those more than, and those less than, fifteen years of age, it was noted that there were 50 with sequelæ in those more than fifteen years of age and only 28 in the other group. The fact that half of our cases were in children accounts to some extent for out percentage of cases showing sequelæ being less than the percentage of other workers.

Table V shows the distribution of the sequelæ:

A study of the classification shows that the greatest number of sequelæ were mental. The next largest group was of motor origin. It is not within the scope of this paper to classify exactly the various types of mental changes. In some instances they were so slight as to be hardly noticeable. In other instances there was a severe mental deterioration that was apparently progressive. How permanent some of the milder sequelæ will be, it is at present impossible to estimate. We have already mentioned that in several instances sequelæ lasting for a year or more eventually disappeared.

TABLE V.

	A. MENTAL	
I.	Blunting of intelligence	30
II.	Nervousness and irritability	13
III.	Defective memory	5
IV.	Changes in character or in disposition	14
V.	Mania or psychosis	2
	B. TROPHIC	6
	C. ENDOCHRINE	5
	D. SENSORY	
I.	Headaches	11
II.	Neuritic pains	6
	E. MOTOR	
I.	Eye changes	
	(*a*) Pupillary	4
	(*b*) Ptosis	2
	(*c*) Strabismus	2
	(*d*) Nystagmus	1
	(*e*) Diplopia	4
	(*f*) Limitation of motion	4
	(*g*) Blurred vision	4
II.	Paralyses or pareses of muscles of face or extremities	13
III.	Parkinsonian syndrome	11
IV.	Tremors, twitches, spasms	11
V.	Epilepsy	1
VI.	Changes in gait	3
	F. MISCELLANEOUS	
I.	Asthenia	9
II.	Digestive disturbances	4
III.	Disturbances of sleep	10
IV.	Speech disturbance	4
V.	Respiratory disturbances	1
VI.	Sphincteric disturbances	1
VII.	Dizziness	3

While we have observed only 11 cases of well-developed Parkinsonian syndrome, certain other cases grouped as asthenia presented some of the features suggestive of an early or mild form of this condition. Perhaps the more common of the motor sequelæ is a paralysis or weakness of some group of muscles of the extremities or face. In one case there was a paralysis of the sternocleidomastoid muscle on one side, thus producing a torticollis.

A general muscular weakness is a rather common after effect. In some cases it is so severe that it would suggest myasthenia gravis, were it not for the history of the previous attack of encephalitis. In the great majority of cases, however, this general asthenia lasts only for several months or a year. This accounts for the small percentage of asthenia as a sequel in our tabulation.

We have also observed some very interesting but rare sequelæ. Four cases of obesity were noted; 1 case developed diabetes mellitus during her convalescence period. It is possible, of course, that this may have been a mere coincidence. We have not noted the report of this complication in the literature, in contrast to diabetes insipidus which has been observed by several writers. Some interesting trophic disturbances have also been observed, such as alopecia, furunculosis, stomatitis, decay of teeth and corneal ulcers.

One patient, an adult, had a very interesting respiratory disturbance. He would get attacks of difficult and labored breathing, lasting from a few seconds to a few minutes. Various types of respiratory difficulties, especially in children, have been referred to in the literature. One patient, a girl aged seven years, showed two years later some of the symptoms that are considered characteristic of multiple sclerosis—nystagmus, scanning speech and staggering gait. The knee jerks were exaggerated. There was, however, no intention tremor.

It is interesting to compare these frequent, diverse and serious sequelæ of epidemic encephalitis with those of poliomyelitis and epidemic meningitis. In the former the sequelæ are certainly sufficiently grave, but they are confined almost entirely to flaccid paralyses. In meningitis only a very small percentage of sequelæ occurs.

For bibliography see the original article.

ENCEPHALITIS ASSOCIATED WITH MEASLES

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Josephine B. Neal and Emanuel Appelbaum

The literature on encephalitis associated with measles is meager and rather indefinite. The ideas with regard to encephalitis have undergone considerable change in the past few years as a result of the intensive study of epidemic encephalitis. There are numerous references to involvement of the central nervous system during or shortly after an attack of measles. In most instances the authors apparently regarded these symptoms as incidental. Only rarely were the symptoms referable to the central nervous system grouped and designated as a definite clinical entity.

It has been recognized for a long time that the various acute infections, as measles, scarlet fever, pneumonia and pertussis, may be followed by encephalitis, most of which have manifested themselves in the form of a cerebral hemiplegia. Particular attention was called to these points by Abt in 1906. While we have seen a few instances of encephalitis following various of the acute infectious diseases, by far the largest number (twelve) have been associated with measles. The first was seen in May, 1918. During the year 1926, particularly, we have seen a comparatively large number (eight). Our experience coincides with that of other clinicians as personally reported to us.

Symptomatology and Diagnosis

The clinical picture is of a highly variable character. The onset of this condition was usually sudden, and developed during or a few days after the attack of measles. In some instances, two weeks elapsed before the appearance of the symptoms referable to the central nervous system. Fever was an almost constant symptom, being present in all but one of our cases. The type of fever was variable. Headache also was almost constantly present. Vomiting was a symptom in only a few cases. Convulsions were present in about half of the cases, and in some instances they were very severe. The most striking of all the symptoms were referable to the mental condition. In some instances there was a mild degree of irritability or apathy. In others there was profound stupor or delirium. Paralysis of various muscles or group of muscles occurred in one-third of our cases. In two instances there was a paralysis of the rectus externus muscle of the right eye, and in one of these cases there was an associated right facial palsy. One patient had a spastic paralysis of the left arm which cleared up in less than a week. The fourth patient had a spastic parlysis of the left arm and a paresis of the left side of the face and the left leg. This patient died after an illness of three weeks, during which period his condition had remained unchanged. Hyperesthesia was fairly common. Muscular twitchings occasionally occurred. General muscular spasticity was a rather common feature. Among the rare symptoms were photophobia, epistaxis, constipation, and retention or incontinence of urine.

The physical signs were chiefly those due to varying degrees of meningeal irritation or increased intracranial pressure. Stiffness of the neck, Kernig's sign, Brudzinski's sign, a bulging fontanel or a positive Macewen sign were noted in a fair proportion of cases. The condition of the reflexes was highly variable. In some instances they were exaggerated and in others they were diminished or absent. In many of our cases, however, the reflexes were normal. The Babinski and its confirmatory reflexes were occasionally present. While ocular symptoms were rare, they were rather striking when present. One patient was apparently blind. Fixed dilation of pupils was present in two cases. There were also two instances of nystagmus and strabismus.

THE SPINAL FLUID

One or more punctures were performed in every case. The fluids usually came out under increased pressure and were clear in all but two instances, which were contaminated with a few red blood cells. Practically all the spinal fluids showed some evidence of abnormality. There was usually a slight or moderate increase in cells, most of which were mononuclears. In most instances there was also a slight or moderate increase in protein content. It should be noted that occasionally a fluid showed an increase in the cytology without showing a parallel increase in the protein. More commonly however, the protein was increased with or without a concomitant increase in the cell content. The sugar was uniformly normal or high. Smears and cultures were all negative for organisms. The accompanying table shows the spinal fluid analyses.

DIFFERENTIAL DIAGNOSIS

It is obvious that all forms of encephalitis present more or less similar clinical pictures. Attention has been called in a preceding paragraph to the association of encephalitis with various acute infections. The diagnosis of our cases of encephalitis associated with measles was based almost exclusively on the manifestations of encephalitis occurring during or shortly after an attack of measles. Indeed, there was nothing in the clinical picture alone to rule out epidemic encephalitis definitely. It may be noted, however, that the onset was more sudden, and, with one exception, the duration was much shorter than is usually the case in epidemic encephalitis.

REPORT OF CASES

Six cases will be described briefly in order to illustrate somewhat the clinical picture and course of the disease.

CASE 1.—G. I., a girl aged 6 years, seen March 20, 1926, had been suffering from measles for one week. Two days after the onset of the measles, she was seized with convulsions, which were followed by apathy deepening into stupor. The examination showed the patient to be acutely ill and still in a semistuporous condition. The temperature was 102 F., the pulse 120, and the respiration 28. There was a questionable Kernig sign. The pupils were rather small but reacted to light. The patellar reflexes were exaggerated. The abdominals were absent. The Babinski reflex was negative but there was a positive Oppenheim sign on the left side. Lumbar puncture yielded 20 cc. of clear fluid under moderately increased pressure. There was a large increase in cells, mostly mononuclears. There was a moderate increase in albumin and globulin. The Fehling's reduction for sugar was normal. Both the smear and the culture were negative for organisms. Lumbar puncture was followed by an immediate improvement, especially in the mental condition. At the end of a week the child had practically recovered. This case seems to illustrate a fairly typical clinical picture and course.

Case 2.—I. D., a boy aged 3 years, was suddenly taken ill with headache and vomiting nine days after an attack of measles. He did not have fever but was apathetic. At times, however, he was quite irritable. The bowels were constipated, but there were no bladder symptoms. Physical examination did not show anything striking. He was, however, definitely apathetic, and had a positive Babinski reflex on his left side. On lumbar puncture, 30 cc. of clear fluid were obtained under increased pressure. The fluid showed a slight increase in cells, mostly mononuclears. The albumin and globulin were also slightly increased, and Fehling's reduction was normal. After an illness of a few weeks the boy made a perfect recovery.

SPINAL FLUID EXAMINATION

Case	Amount of Fluid Cc.	Appearance	Cytology	Bacteriology	Albumin	Globulin	Sugar
1	20	Clear	Large increase of mononuclears	Negative	++	++	+++
2	30	Clear	Slight increase of mononuclears	Negative	+	+	+++
3	15	Blood-tinged with web	Mostly red blood cells	Negative	+1	+1	+++
4 (*a*)	30	Clear	3 mononuclears	Negative	++	+	+++ (57 mg.)
(*b*)	..	Clear	12 mononuclears	Negative	+	+	+++
(*c*)	15	Clear	8 mononuclears	Negative	±	±	+++
5	24	Clear	3 mononuclears	Negative	+	..	+++
6	30	Clear	15 mononuclears	Negative	+	+	+++ (84.2 mg.)
7	15	Clear	Normal	Negative	+	+	+++
8	20	Clear	87 mononuclears	Negative	+1	+1	+++
9	20	Clear	Slight increase of mononuclears	Negative Negative	±	±	+++
10	15	Clear with web	Large increase of mononuclears	Negative	+1	+1	+++
11	15	Clear	Normal	Negative	±	±	+++
12	5	Blood-tinged	Red blood cells		±	±	+++

This was undoubtedly a mild case of encephalitis following an attack of measles. It should be noted that there was an interval of nine days between the measles and the encephalitis.

Case 3.—E. H., a boy aged 7 years, was taken ill suddenly, April 27, 1926, with a headache, convulsions, and a fever of 102 F. Shortly after the onset of this illness he developed a spastic paralysis of the left arm. Six days prior to this illness he had an attack of measles. When we saw him, April 30, he was quite apathetic. His temperature was 100.4 F., pulse 120 and respiration 28. Hyperesthesia was marked. There was a positive Kernig sign on the right side and also a positive Brudzinski sign. The Babinski sign was questionable. There was a definite spastic paralysis of the left upper extremity.

Lumbar puncture yielded 15 cc. of slightly blood-tinged fluid, which showed a moderate increase in albumin and globulin and a normal Fehlig's reduction. Smear and culture were negative for organisms.

May 3, the child had completely recovered. The paralysis of the left arm had cleared up, and he seemed to be perfectly normal in every way.

This case illustrates some interesting points. It shows that one cannot be guided by the severity of the case in determining the prognosis. The child certainly was very ill and yet made an uneventful recovery. It is also obvious that the total duration of the illness may be very brief.

Case 4.—W. F., a boy aged 20 months, had measles January 11, 1926. This was followed by bronchitis, for which he was admitted to a hospital January 23. On January 26 he developed weakness and spasticity of the left upper and lower extremities. After a few days the left arm became definitely paralyzed, and there also developed paresis of the left half of the face. On examination, the child was very apathetic. When aroused, he was irritable. The neck was slightly rigid. The pupils were small and reacted to light. The deep reflexes were normal on the right side and exaggerated on the left side. The abdominals were absent on both sides. There were no pathologic reflexes. There was a spastic paralysis of the left arm and paresis of the left side of the face and left lower extremity. The fundi were slightly hazy. There was a low irregular fever throughout the illness.

Three lumbar punctures were performed. The first two fluids did not show increase in cells; there was a slight increase in albumin and globulin and a normal Fehlig's reduction. Smear and culture were negative for organisms. The third fluid was normal in every respect. Both the spinal fluid and the blood Wasserman test were negative.

The child was ill for about three weeks, during which period his condition remained unchanged. He died, February 11.

Although the clinical course was not by any means stormy, the result was fatal.

Case 5.—T. S., a boy aged 2½ years, seen January 26, 1926, had developed measles on the 15th. Five days later meningeal symptoms developed. These consisted of severe convulsions, vomiting and fever up to 105 F. There was also apathy and incontinence of urine.

When examined, January 26, there was no stiffness of the neck, Kernig sign or Brudzinski sign. The pupils were equal and reacted to light, but the child was apparently blind. He was also unable to speak. There was a right facial palsy and also a paralysis of the right external rectus muscle. The deep and superficial reflexes were abolished and there were no abnormal reflexes. The temperature was 105 F., the pulse 120 and the respiration 40.

During the examination, convulsions occurred. These involved both arms and the face, but not the legs. Several spinal fluids were examined during the illness. The results were normal except for a slight increase in protein. The child died, January 27.

This case illustrates an unusually severe type of the disease.

Case 6.—S. R., a boy aged 5 years, had suffered from an attack of measles, from which he had made a good recovery and had been well for three days. On the fourth day of his convalescence (May 3, 1926) he suddenly had a convulsive seizure, and then went into a deep coma. This was accompanied by high fever. He apparently had a slight headache for a short period preceding this explosive onset.

On examination about five hours after the onset, the child appeared so ill that it seemed as if a fatal outcome were imminent. He was in a deep stupor and was markedly cyanosed. He had twitchings of the right side of the face and the right arm. There was no rigidity of the neck, Kernig sign or Brudzinski sign. There was a positive Babinski reflex on the right side. All the deep and superficial reflexes were present. There was a nystagmoid movement of the eyes to the left. The pupils were widely dilated and did not react to light. The lungs presented the signs of pulmonary edema. The temperature was 104 F., and the pulse 120; the respirations were Cheyne-Stokes in character.

A lumbar puncture performed shortly after the onset yielded a normal fluid. The following day the child was definitely improved. The temperature dropped to normal and the child regained a certain degree of consciousness. This improvement, however, was of very short duration. Within twenty-four hours the temperature rose and the child lapsed into deep coma again.

May 6, a second lumbar puncture yielded 30 cc. of clear fluid. This showed 15 mononuclears, a slight increase in protein, and a rather high sugar content (84.2 mg. per hundred cubic centimeters). There were no organisms on smear or culture.

Within two or three days the clinical picture changed decidedly. The child's mind appeared blank, and his eyes showed a meaningless and aimless stare. At the same time he was extremely restless and hyperactive. He constantly tossed about and made repeated efforts to scale the crib. He vigorously and violently resisted all attempts to restrain him. He showed definite negativism, and refused to take nourishment. As a result gavage feeding was resorted to. Except for an occasional shrill cry, he was speechless. He had a low, irregular fever. A few days later another complication appeared. He began to have frequent petit mal seizures. This hectic clinical picture persisted for about two weeks. Then the temperature dropped to normal and he began to take food by mouth. The restlessness abated somewhat. The petit mal seizures, however, persisted, though they became less frequent. He began to show some interest in his surroundings, and would follow his mother around. At the same time he began to utter a few words. At this point his progress ceased and for the succeeding few months his condition remained practically unchanged. At the last examination he was in excellent condition physically but seemed to have improved very little mentally. He was still restless and apparently unaware of his surroundings. Some of his memory associations, however, have been restored. He would frequently sing an isolated line of an old favorite song and would repeatedly call out his teacher's name. He would also call for his mother or father occasionally. His speech was thus limited to a few isolated phrases and words, pronounced rather indistinctly. He did not seem to undertand when spoken to, nor did he seem to be able to interpret objects. The petit mal seizures still recurred at varying intervals. At that time it seemed to us that the boy was destined to remain a mental defective.

We have cited this case in detail in order to illustrate some interesting and important points. This was by far the most severe and malignant form of the disease which did not end in death. It was the only case that ran a chronic course. A third point of importance is the apparent extensive injury to the brain as shown by the present clinical picture. Although it is difficult to give a definite and accurate prognosis, it seems as if the mental changes in this case will be permanent.

COMMENT

Of the twelve patients that we have seen with this disease, three died, a mortality of 25 per cent. Seven of the nine who recovered were followed up. All except the one just cited made a complete recovery.

It seems fairly certain that an acute encephalitis is occasionally closely associated with various acute infections, especially measles. Whether or not this encephalitis bears any relation to the epidemic form it is at present impossible to say. It is true that all these cases that we have recorded have occurred since 1918. It is possible that physicians have taken more interest in such conditions on account of the discussion stimulated by the prevalence of epidemic encephalitis. It is obviously impossible to make a definite statement as to the etiology and pathology of this condition. The subject certainly merits further study.

For bibliography see the original article.

MENINGITIS

Distribution According to Age and Etiology

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Josephine B. Neal

In the recent literature a number of interesting papers have appeared dealing with the incidence of meningitis in early infancy, particularly in the first two or three months of life. A study of these papers reveals the fact that such cases are comparatively rare. Three very valuable contributions to this subject are the articles by Barron, in 1918; by Root, in 1921, and by Cooke and Bell, in 1922. These papers contain extensive reviews of the literature and complete bibliographies. No attempt will be made in this paper to review the literature again, but references will be made to the statistics collected therein for purposes of comparison with our own tables.

Since 1910, when the meningitis division of the department of health of New York City was established by Dr. W. H. Park, there has been an opportunity for the members of that division to study a rather large number of cases of meningitis. During that time there has been no epidemic of meningococcic meningitis. Therefore Table 1 represents what may perhaps be considered a normal distribution of meningitis in New York City from an etiologic standpoint. It shows 1,523 cases of meningitis arranged according to age and the more common etiologic agents.

In addition, we have seen the following cases due to more unusual organisms: Friedländer's bacillus, two patients, one aged 2 years and one adult; B. *pyocyaneus,* one patient, aged 7 years; *Micrococcus catarrhalis,* one patient, aged 7 weeks; Streptothrix group, one patient, aged 12 years; torula, one patient, aged 16 years.

The cases due to mixed infections are as follows: meningococcus and staphylococcus, one patient, aged 9 years; meningococcus and B. *paratyphosus* B, one patient, aged 7 months; *Streptococcus hemolyticus and Staphylococcus albus,* one patient, aged 3 months; streptococcus, pneumococcus and staphylococcus, one patient aged 7 years; streptococcus (nonhemolyzing) staphylococcus and unidentified gram-amphophilic coccus, one patient, aged 8 years; a mixed infection containing six organisms, which were never satisfactorily classified, the only identified pathogenic organism belonging to the *B. coli* group, one patient, aged 16 years. This makes a total of 1,535 cases.

TABLE I.
DISTRIBUTION OF MENINGITIS ACCORDING TO AGE AND ETIOLOGY

Age	Tubercle Bacillus	Meningococcus	Pneumococcus	Streptococcus	Influenza Bacillus	Staphylococcus	Colon Bacillus	Total
3 months	5	24	3	11	2	0	3	48
3 to 6 months	21	58	4	6	8	0	0	97
6 to 12 mos.	78	68	11	3	14	2	0	176
No. cases under 1 year	104	150	18	20	24	2	3	321
1 to 2 years	178	79	8	9	14	2	1	291
2 to 3 years	80	36	9	6	6	1	0	138
3 to 5 years	99	80	6	11	1	2	0	199
5 to 10 years	96	105	16	20	3	0	1	241
10 to 20 years	57	101	7	5	3	2	0	175
Over 20 years	48	74	22	12	0	2	0	158
Totals	662	625	86	83	51	11	5	1,523

Of this number, fifty were 3 months old or younger. It is interesting to observe that twenty-four of these cases were due to the meningococcus, eleven to the streptococcus, five to the tubercule bacillus, three to the pneumococcus, three to the *Bacillus coli*, two to *Bacillus influenzae*, one to *Micrococcus catarrhalis*, and one to mixed *Streptococcus hemolyticus* and *Staphylococcus albus*. This is in marked contrast to the report of Barron, who compiled from the literature thirty-nine cases of meningitis in infants under 3 months of age, and found that thirteen were due to *Bacillus coli* and only five to the meningococcus. He reported another case also due to *Bacillus coli*. In many of the cases reported in the literature, the diagnosis was made at necropsy and there is a possibility, perhaps, that the *Bacillus coli* isolated may have been an invader just before or just after death, and not the specific cause of the meningitis. Since Barron's article, other cases of meningitis in young infants, due to members of the colon group, have been reported by Greenthal, Smith and Aberd, Sherman, and Cooke and Bell. In view of our own experience, covering a relatively large number of infants under 3 months of age, we must consider *Bacillus coli* as a comparatively rare cause of meningitis in infants.

That the streptococcus is a not uncommon cause of meningitis in infants is shown by the fact that eleven of the fifty cases were due to this organism. Similar findings have already been reported by Holt and Koplik.

The only reference we have been able to find to tuberculous meningitis in early life is by Holt, who reported three cases in infants under 3 months of age in a series of 218 children under 3 years of age.

The number of cases due to the meningococcus, twenty-four, is surprisingly large, compared with other reports. Cooke and Bell reported in 1922, that they were able to find only twelve cases in the literature of the last five years. To these they added four cases, and Root's case also should be added. Root compiled from the literature thirty-four cases of meningitis in infants under 2 months of age: due to the colon bacillus, eleven; to the staphylococcus and streptococcus, nine; to the meningococcus, six; to the pneumococcus, four; to *Bacillus lactis-aerogenes*, one; to *Bacillus mucosus capsulatus*, one; to *Bacillus pyocyaneus*, one, and to *Micrococcus catarrhalis*, one.

For comparison with these figures, we have tabulated our own cases of infants under 2 months of age (Table 2).

TABLE 2.

CASES OF MENINGITIS IN INFANTS UNDER TWO MONTHS OF AGE

	Age and Number	Total
Streptococcus	6 weeks, 3; 7 weeks, 2; 8 weeks, 1	6
Meningococcus	3 weeks, 2; 7 weeks, 1	3
B. coli	2 weeks, 1; 5 weeks, 1	2
B. influenzae	2 months, 2	2
Pneumococcus	2 months, 1	1
Micrococcus catarrhalis	7 weeks, 1	1
Total		15

It will be seen that our youngest case was that of *Bacillus coli* meningitis in an infant, aged 2 weeks. Two cases due to the meningococcus were in infants aged 3 weeks.

It is generally recognized that meningitis is essentially a disease of childhood. It is, perhaps, not generally realized that so large a proportion of cases occur in children during the first year of life. It will be seen in Table 1 that far more cases of meningococcic meningitis are found in children in the first year than in any one year; indeed, that the number in the first year is nearly twice as great as that in the second year, and more even than in the five year group from the fifth to the tenth years. In all other forms of purulent meningitis also, there are more cases in infants in the first year than in any other year. In tuberculous meningitis the number of children in the second year of life exceeds that in the first year; but, taking all forms of meningitis together, the greatest number is found in the first year.

Adding together the cases of patients under 10 years of age, it will be seen that 1,190, or more than 78 per cent. of the total number, occur in this age group.

As regards the etiologic distribution, it will be noted that more cases of tuberculous than of meningococcic meningitis have been seen. As was stated earlier, since 1910 there has been nothing approaching an epidemic of meningococcic meningitis in New York City. Indeed, since the spring of 1919, the number of cases has been extremely small. After the meningococcus, the pneumococcus will be seen to be the most common cause of purulent meningitis. Eighty-six cases were due to this organism. Eighty-five cultures from the spinal fluids of patients who had pneumococcus meningitis have been typed. A part of these were fluids sent in by physicians from patients that we did not see. Most of the patients, however, were seen by us. The results were as follows: Type I, eighteen; Type II, twenty-two; Type III, fifteen; Group IV, thirty.

The number of cases of meningitis due to the streptococcus, eighty-three, almost equals those due to the pneumococcus. *Streptococcus viridans* very rarely causes streptococcic meningitis. Practically all the cases are due to one of the hemolyzing streptococci.

Bacillus influenzae is the fourth most common cause of purulent meningitis, causing fifty-one cases in our list, with the staphylococcus and *Bacillus coli* ranking fifth and sixth, respectively. The number of cases caused by these last two organisms is very small.

Practically any of the pyogenic organisms may occasionally invade the meninges, and set up a meningitis. The same is true of the higher organisms, such as members of the streptothrix group, and the pathogenic yeasts, of each of which we have one case in our list. It will also be seen that various organisms may combine to set up mixed infections, though, in our experience, mixed infections are comparatively rare.

SUMMARY

In a list containing 1,535 cases of meningitis arranged according to age and etiology, it is seen that:

1. With the exception of tuberculous meningitis, more cases of meningitis occur in the first year of life than in any other year. The number of cases of meningococic meningitis in the first year of life far exceeds those in any other year.

2. The greatest number of cases of tuberculous meningitis is found in the second year of life.

3. In times when there is no epidemic, the number of cases of tuberculous meningitis equals or exceeds the number of cases of meningococcic meningitis.

4. After the meningococcus, the pneumococcus and the streptococcus are the most common causes of purulent meningitis, followed by the influenza bacillus, the staphylococcus and *Bacillus coli,* in the order named. Cases due to the last two organisms are comparatively rare.

5. Other pyogenic organisms occasionally cause a meningitis, and, more rarely, members of the higher group of organisms, such as the members of the streptothrix group and the pathogenic yeasts. Mixed infections are rare.

For bibliography see the original article.

EPIDEMIC MENINGITIS

A Study of More Than Six Hundred and Fifty Cases, with Especial Reference to Sequelae

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Josephine B. Neal, Henry W. Jackson and Emanuel Appelbaum

While epidemic meningitis is by no means a rare disease, the number of cases occurring when there is no epidemic is small. It seems worth while, therefore, to call attention to certain facts in regard to the disease based on a study of meningeal conditions in New York covering a period of sixteen years. During this time there has been no epidemic, so that the data show what occurs under normal conditions.

We have been impressed with the fact that most of the laity and many physicians think that patients recovering from epidemic meningitis are liable to be seriously handicapped, especially in their mental development. For this reason we have followed up as many as possible of our patients who recovered to learn what percentage of cases show after-effects, and the nature of them.

Epidemiology

Epidemic meningitis is apparently a disease to which most persons have a natural immunity. In our study we have on record only fourteen instances in which more than one case occurred in the same family. As most of these cases have been in crowded districts, it is obvious that these 654 patients have exposed to the infection several thousand persons. In spite of this we have not found cases occurring in neighboring apartments, except once. In this instance, a man residing in an upper apartment developed epidemic meningitis at about the same time as a case occurred in another family occupying a lower apartment in the same building.

It not infrequently happens that epidemic meningitis is superimposed on some other acute infection, particularly pneumonia or influenza.

In this locality by far the greatest number of cases have occurred during the first six months of the year. Table 1 shows the seasonal distribution.

A study of the distribution by sex of 650 cases shows a preponderance of males, nearly in the ratio of 3:2, as indicated in table 2.

TABLE 1.

SEASONAL DISTRIBUTION OF EPIDEMIC CEREBROSPINAL MENINGITIS

Year	January, February, March	April, May, June	July, August, September	October, November, December	Total
1910	..	..	1	4	5
1911	3	9	6	4	22
1912	8	6	4	7	25
1913	9	10	1	4	24
1914	25	11	5	10	51
1915	14	10	5	6	35
1916	22	28	10	15	75
1917	19	45	9	12	85
1918	51	46	19	20	136
1919	20	19	9	9	57
1920	13	5	4	5	27
1921	15	9	5	7	36
1922	13	8	2	5	28
1923	6	6	3	3	18
1924	3	6	3	1	13
1925	5	1	5	6	17
Total	226	219	91	118	654
Seasonal distribution by percentage	34.6	33.5	13.9	18	

TABLE 2.

SEX DISTRIBUTION OF EPIDEMIC CEREBROSPINAL MENINGITIS

1925 Inclusive

	Male	Female	Total	Unknown
3 months	16	6	22	1
3 to 6 months	37	26	63	..
6 to 12 months	56	24	80	..
1 to 2 years	50	27	77	1
2 to 3 years	15	22	37	..
3 to 5 years	42	39	81	..
5 to 10 years	50	56	106	2
10 to 20 years	57	44	101	..
20 to 30 years	40	14	54	..
30 to 40 years	7	11	18	..
40 to 50 years	3	7	10	..
50 to 60 years	0	1	1	..
Total	377	277	650	4
Percentage	57.5	42.5		

A study of the age distribution reveals some interesting and instructive facts. A glance at table 3 will readily show that epidemic meningitis is largely a disease of infancy and early childhood. A far larger number of cases occur during the first year of life than in any other one year. This, incidentally, raises the mortality rate, as this disease is especially fatal during early infancy.

TABLE 3.

AGE DISTRIBUTION

3 months	23
3 to 6 months	63
6 to 12 months	80
Total under 1 year	166
1 to 2 years	78
2 to 3 years	37
3 to 5 years	81
5 to 10 years	108
10 to 20 years	101
20 to 30 years	54
30 to 40 years	18
40 to 50 years	10
50 to 60 years	1
Total	654

ETIOLOGY

The causative agent of epidemic meningitis is the meningococcus, the presence of which in the spinal fluid must be proved by culture before an absolute diagnosis can be made. It is probable that there is a brief invasion by the organism into the blood in practically all cases of epidemic meningitis. It is now commonly accepted that the organisms usually enter the blood stream through the mucous membrane of the nasopharynx, and then localize in the meninges. While the presence of meningococci in the blood is usually transient, in a certain type of case, the septicemic, they may persist in the blood stream for a considerable period of time, either before or after the meningeal localization. It occasionally happens that the meningococci remain in the blood and do not reach the meninges, thus producing a true meningococcemia.

SYMPTOMATOLOGY

While many writers divide epidemic meningitis into a variety of classes, an adequate conception of the disease may perhaps be expressed by presenting four clinical pictures; that of the most common type occurring in older children and adults; that occurring in infancy; the septicemic type, and the fulminating type. The clinical picture in all is modified by the severity of the infection and the resistance of the individual.

The Usual Picture in Older Children and Adults.—The classic picture of epidemic meningitis in older children and adults is fairly familiar to all. There is a sudden onset, with headache, vomiting and fever of an irregular type. Convulsions may occur, especially in children. Hyperesthesia is fairly common. Delirium is not uncommon. There is a petechial eruption in 12 or 15 per cent. of cases, which soon fades. An herpetic eruption is fairly frequent. The bowels are usually constipated, and there is sometimes retention of urine. Within the first twenty-four to forty-eight hours, stiffness of the neck and the Kernig and Brudzinski signs appear. Early in the disease, the tendon reflexes are likely to be exaggerated and equal. Later they may become unequal, diminished and finally lost. The reaction of the pupils to light follows the same course. In children, a positive Macewen sign indicating increased intraventricular pressure can usually be detected. Strabismus occasionally occurs, more frequently in children than in adults.

The course of the disease in a treated case depends on how early the serum is administered and the response of the patient to treatment.

There are four courses which the disease may pursue: 1. The disease may run an acute course, the patient either dying or recovering after a few doses of serum. 2. The disease may be greatly prolonged, so that twenty or more doses of serum may be given before recovery or death finally takes place. 3. The disease may relapse after apparent recovery, indicating, perhaps, some focal or walled off infection that breaks down. In this type of case recovery may take place. We have comparatively few cases belonging to this class. 4. There is a class in which there is blocking at the base. This type, with few exceptions, is fatal.

The Clinical Picture in Infancy.—It has already been shown in table 3 that more cases occur in the first year of life than in any other one year. It is at this age that the mortality is highest and the diagnosis most difficult.

In infancy we do not see early the stiffness of the neck, Kernig's sign and the changes in reflexes that characterize the disease in older children and adults. The onset is less sudden. There is often vomiting and practically always some intestinal disturbance. There is an irregular fever. The gastro-intestinal condition does not respond to the usual method of treatment. Convulsions often occur early, but these are so common in other diseases of infancy that they are not diagnostic. Usually ten days or two weeks have elapsed before symptoms develop that direct the physician's attention to the central nervous system. These may be the developmnt of strabismus, a fairly well defined rigidity of the neck or severe convulsions. Much earlier, a bulging of the fontanel might have been detected if an examination of the child had been made when not crying. The pupillary and patellar reflexes are often normal when the case is well advanced. Certainly, an irregular fever and a gastro-enteritis that does not respond readily to treatment especially when there is a bulging fontanel, are sufficient indications for an early lumbar puncture. It is in these young children that a walling off at the base with a resulting dry tap most frequently occurs.

The Septicemic Type.—This type of meningitis is quite rare among sporadic cases. In certain of the army camps during the war there was a very large percentage of this type of case. The early picture is that of a septicemia rather than a meningitis, the meningeal symptoms developing five or six days or more after the onset. A profuse and rapidly spreading macular or purpuric rash is an almost constant feature. The rash usually persists for some time. Other indications of a generalized blood infection, such as joint involvements, may be present even before the meningeal symptoms.

The Fulminating Type.—This form is rare. It is characterized by a violent onset. The patient is usually seized with severe headache and vomiting, and speedily loses consciousness. The course is very brief, death supervening within from twenty-four to forty-eight hours. It sometimes happens that the patient dies in an even shorter period. There are cases on record in which the patient retired in apparently good health and was found dead in the morning. The importance of this from a medicolegal standpoint must be borne in mind. It may be of interest to note that this form of meningitis is most likely to occur in persons of the status lymphaticus type.

DIAGNOSIS

There is a large number of diseases which may in some respects simulate epidemic meningitis. The following are the more important conditions that may cause confusion in the diagnosis: other forms of purulent meningitis; occasionally, tuberculous meningitis; acute anterior poliomyelitis (especially the nonparalytic type); epidemic encephalitis; brain abscess; sinus thrombosis; cerebrospinal syphilis; tetanus; meningeal or cerebral hemorrhage; uremia; meningism associated with acute infections, such as gastroenteritis, acidosis, tetany and typhoid; typhus fever; bacterial endocarditis; purpura hemorrhagica; the acute leukemias, and certain forms of drug and metallic poisonings.

Space does not permit a discussion of the differentiated points of diagnosis. An absolute diagnosis of epidemic meningitis depends on the demonstration by culture of the meningococcus in the spinal fluid. A presumptive diagnosis, however, may be made in cases showing the characteristic changes of meningitis in the spinal fluid, and responding to serum treatment, even though the presence of meningococci has not been demonstrated.

LABORATORY OBSERVATIONS

The blood shows a varying increase in the leukocyte count, and there is a polymorphonucleosis. It has been quite definitely proved that there is usually an early transient bacteremia. As has already been stated in the septicemic type of the disease, the meningococci persist in the blood for a comparatively long time.

The examination of the spinal fluid is by far the most useful aid in diagnosis. Netter states that in the first twenty-four hours the fluid is practically almost clear and mononuclears predominate. We have seen very few cases so early as this. In the early stages of the disease the fluid is increased in amount and shows varying degrees of turbidity. The cells are greatly increased and the polymorphonuclears predominate.

The stained smear shows varying numbers of gram-negative diplococci, both intracellular and extracellular. The protein content is moderately to greatly increased. The sugar content may be normal early in the disease, but later it practically always is diminished or absent. With improvement in the clinical picture there is usually a decided rise in the amount of sugar. We regard this as a valuable prognostic point.

On suitable mediums, the meningococci develop in twenty-four hours or more. Their identity may be proved by agglutination with meningococcus serum and the fermentation test. A definite diagnoses cannot be made from the smear alone, as meningitis may be caused by other gram-negative cocci; moreover, the gram-positive cocci are sometimes easily decolorized in the smear.

In one instance, when the diagnosis was not made during the acute stage and serum was not administered, the patient's serum showed well defined complement fixation with the meningococcic antigen. We do not regard this as a valuable diagnostic aid, as it has failed in other cases.

TREATMENT

We have always favored a conservative method in treatment. We ordinarily give serum every twenty-four hours, injecting 20 cc. by gravity intraspinally if as much or more of the fluid has been obtained. In certain instances, when only a small amount of fluid is obtained, we inject more than the amount of fluid withdrawn, provided the serum runs in easily by gravity and no untoward symptoms result. We continue the daily intraspinal injection of serum until the fluid is sterile. After this the injection of serum is determined by the clinical picture. It is often necessary to do one or more lumbar punctures during convalescence for relief of pressure without administering serum. In the average case with recovery, from six to ten doses of serum are required. It is rarely safe to give less than four injections. In very severe cases we often give the second and third injections at eighteen hour intervals. In the septicemic type of case in adults, we administer the serum intravenously as well as intraspinally. We do not, however, approve of the intravenous method of serum administration in children, concurring in this with the opinions of Netter and Blackfan. Incidentally, the septicemic type is rare in children.

During the last few years there has been a trend toward a more intensive method of treatment. Advocates of this method give serum every twelve or even every eight hours intraspinally, and also give it intravenously and intra muscularly in every case. In order to compare the results of this method of treatment with our own, we studied the records of a children's service at an excellent hospital in the city from January, 1918, to October, 1921. During this period, seventy-nine cases were treated at that hospital by the more intensive method, with a mortality of 54 per cent. During the same period we treated 254 cases, with a mortality of 23 per cent. This seemed to us very strong proof that there is no advantage in the more heroic method of treatment. Much attention has been called to the favorable results following the intravenous use of serum in certain of the army camps in which the septicemic type predominated. It is important, therefore, to note that successful results were obtained by the intraspinal method of treatment alone in those camps in which the septicemic type of case did not prevail. In a personal communication, Dr. Harold E. Smith stated that at Camp Mills eighty cases were treated only by intraspinal injections with a mortality of 10 per cent.—an excellent result. It certainly seems to us that the enthusiasm for administering serum intravenously in cases other than the septicemic type is ungrounded.

It quite frequently happens that in infants there is a blocking at the base of the brain, so that little or no fluid can be obtained by lumbar puncture. In these cases it is necessary to resort to ventricular puncture for the withdrawal of fluid and the injection of serum. By this method the ventricular fluid is usually rendered sterile. However, in all but two instances the adhesions have persisted with resulting hydrocephalus, and death has followed. The ventricular puncture is usually performerd daily in alternate ventricles. When adhesions occur along the spine a cisternal puncture has been resorted to for relief of pressure. As we have been afraid of pressure on the medulla, we have not given serum by this method. In young babies the ventricular puncture is safer than the cisternal puncture. Reports have been made to us of hemorrhage following cisternal puncture in infants. In older children and in adults we have resorted to trephine in order to do a ventricular puncture. The results have been uniformly unsatisfactory. It is important to note that failure to obtain fluid by lumbar puncture does not always indicate adhesions, as it sometimes happens in these cases that a considerable amount of serum may be introduced readily by gravity. In a number of instances there has been recovery in cases of this type. A recent article[1] emphasizes the value of both ventricular and cisternal punctures in advanced meningococcus meningitis. As stated, our results have been decidedly unsatisfactory in the number of cases treated by more radical methods. It is to be noted that the author of the article referred to cites very few cases (and only one of his own) on which to base his opinion. We have on record many similarly obstinate cases treated only by spinal puncture in which the results were equally brilliant and successful. However, we appreciate that in rare instances ventricular or cisternal punctures may be valuable adjuvants to the spinal method.

In cases that have become chronic, we have used autogenous vaccine sometimes with apparently satisfactory results, both intraspinally and subcutaneously.

The general treatment is largely symptomatic. The three points that must always be borne in mind are that the patient must be kept quiet by adequate sedatives; he must have sufficient nourishment, and attention must be paid to elimination, as constipation is usually present and retention may occur. The patient should be kept in a quiet, dark room with proper ventilation.

MORTALITY

A certain number of cases were omitted from the mortality statistics chiefly because we were unable to obtain cooperation in carrying out the treatment. Our mortality for 627 cases is nearly 30 per cent. Certain factors have operated to make this mortality so high. The large percentage of children under 1 year of age is an important factor. Most of our patients had been ill several days and some as long as two or three weeks before we were called in consultation. A certain number of patients had pneumonia or some complication not directly connected with the meningitis which was a contributing factor in the cause of death.

In certain years the mortality has been very satisfactory; for example, from July 1, 1916, to July 1, 1917, eighty-five patients were treated, with a mortality of 23.5 per cent.; from July 1, 1917, to July 1, 1918, 112 patients were treated, with a mortality of 19 per cent.; from July 1, 1918, to July 1, 1919, seventy-eight patients received treatment, with a mortality of 20.5 per cent. Table 4 shows the mortality by age groups.

TABLE 4.
MORTALITY BY AGE
1925 Inclusive

	Recovered	Fatal	Total	Mortality, per Cent.
Under 1 year	83	72	155	46.5
1 to 2 years	51	21	72	29.2
2 to 5 years	92	23	115	20
5 to 10 years	87	22	109	20.2
10 to 20 years	74	23	97	23.7
20 to 30 years	35	18	53	34
Over 30 years	18	8	26	30.8
Total	440	187	627	29.8

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[1] Peet, M. M.: Advanced Meningococcus Meningitis, J. A. M. A. 86: 1818 (June 12) 1926.

SEQUELAE

Many of the impressions in regard to the sequelae of epidemic meningitis seem to be based on the history obtained from parents who are prone to ascribe the cause of some defect, especially mental, to a previous illness. In view of the many diseases that may be confused with epidemic meningitis, unless a careful study of the spinal fluid is made, it has seemed to us important to determine the final outcome in a fairly large number of cases definitely diagnosed.

Our study shows that the great majority, 82 per cent., make a complete recovery. About 18 per cent. of the cases do show sequelae, and these are often of a serious nature. It is important to note that the defects for the most part develop during the acute stage of the disease and are the direct and immediate result of the inflammatory process. This is in marked contrast to the sequelæ of epidemic encephalitis which develop months or even years after the more acute process has subsided.

The most important and frequent of the sequelæ is deafness. This is usually bilateral, complete and permanent. In a certain number of cases, however, there is partial or complete recovery from the deafness. In the younger children the deafness was accompanied by mutism. Once there was mutism not associated with deafness. Another patient had halting speech.

Defects of vision are of rarer occurrence. They were found in seven instances. In one case there was bilateral defective vision secondary to hydrocephalus. In the remaining six there was an iridochoroiditis which terminated in a panophthalmitis resulting in complete loss of vision except in one instance, in which, after a short period of complete blindness, the patient regained power to perceive light. It should be noted that the blindness was unilateral in all but one case.

There is a widespread impression that epidemic meningitis is liable to be followed by defective mental development. Our experience does not bear this out. In only eight instances was there any indication of residual disturbances in mentality or intelligence. Four patients, one of whom suffered from hydrocephalus, were definitely subnormal mentally. Three patients complained of nervousness, which may or may not have been the result of the meningitis. One patient became confused occasionally in conversation, but he was able to do clerical work.

Eleven patients showed some form of paralysis, the detailed distribution of which may be noted in the table. These paralytic patients showed a tendency to improve in many instances, and some of them gave promise of complete recovery in the future.

In those patients suffering from headache or general weakness, it was difficult to decide to what extent the meningitis was actually responsible for these conditions.

Rarer but nevertheless important sequelæ were also encountered. In two instances there were sphincteric disturbances—one rectal and one vesical. One patient developed a pachymeningitis nearly two years later. Another patient developed a complete transverse myelitis, which had its inception early in the convalescent period. Table 5 gives a detailed list of the sequelæ.

The total number of cases followed up was 337. In 276 of these, or 81.9 per cent., there was complete recovery, while 61, or 18.1 per cent., showed sequelæ. In several instances there was more than one sequela.

TABLE 5.
SEQUELAE

		Number	Per Cent.
1. Impairment of hearing		26	7.7
(a) Totally deaf	21		
(b) Partially deaf	5		
One was totally blind in one eye			
One had headaches			
2. Paralyses		11	3.2
(a) Four had strabismus			
(b) Right leg and thigh and ankylosis of spine, lumbar region			
(c) Right arm			
(d) Two fingers of right hand			
(e) Right leg			
(f) Strabismus and facial paralysis			
(g) Right hand slightly			
(h) Left arm and leg			
3. Mental disturbances		8	2.3
(r) One hydrocephalic; weakness of legs and ataxic gait; some defect of vision; retarded development			
(b) Two showed poor mental development			
(c) Three, nervousness			
(d) One mentally confused occasionally			
(e) One poor progress in school, forgetfulness and headaches			
4. Defects of vision (5 plus 2 already listed)		7	2.1
(a) One blind in both eyes, nervous, fretful			
(b) Three blind in one eye			
(c) One defective vision in one eye and also nervousness; one case has already been classified under defective hearing, and a second under metal defects			
5. Headaches		3	0.9
6. Speech defects		2	0.6
(a) One had halting speech			
(b) One mute but could hear			
7. Sphincteric disturbances		2	0.6
(a) One rectal			
(b) One difficulty in voiding			
8. General weakness		2	0.6
(a) One unable to work			
(b) One complained of pain and stiffness of back and general weakness			
9. Pachymeningitis nearly two years after illness		1	0.3
10. Transverse myelitis with rectal and vesical involvement and paralysis of both lower extremities		1	0.3

EPIDEMIC MENINGITIS IN THE FIRST THREE MONTHS OF LIFE

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The literature on epidemic meningitis in infancy is meager. Studies of meningitis in early infancy with reviews of the literature have been made by Barron, Root, and Cooke and Bell, and an interesting case of epidemic meningitis has been recently reported by Sturtevant. In view of the undoubted fact that a considerable number of cases are never reported, it does not seem important to make a very careful study of the literature with an attempt to collect the number of cases.

It appears to be a general belief that all forms of meningitis are rare in early infancy. Barron writes, "Although meningitis is a disease quite common in childhood, it is one that is extremely rare during the early months of infancy." Sturtevant states: "Meningococcus meningitis is an extremely rare disease in early infancy." Similar statements are made by Root and by Cooke and Bell. It is our impression, from our own experience, that meningitis occurs in infants more often than is generally believed.

During the sixteen years that we have been studying meningitis and meningeal conditions in New York City, we have had the opportunity of seeing a fairly large number of cases of meningitis of all types in young babies. It seems worth while, therefore, to report our experience. We are limiting our study to those cases occurring during the first three months of life.

During this time we have seen fifty-four cases with the etiologic distribution shown in table 1. A glance at the table shows the great preponderance of cases due to the meningococcus. This group is also the most important from the standpoint of treatment.

TABLE 1.

ETIOLOGIC DISTRIBUTION OF EPIDEMIC MENINGITIS DURING FIRST THREE MONTHS OF LIFE

Meningococcus	Streptococcus	Pneumococcus	Tubercle Bacillus	Colon Bacillus	Influenza Bacillus	Micrococcus Catarrhalis	Total
25	11	7	5	3	2	1	54

SYMPTOMATOLOGY

It is not generally recognized that the symptoms of meningitis in infancy are quite different from those in older children and in adults. Since the symptoms of all purulent forms of meningitis are practically the same, we will discuss in detail the symptoms of epidemic meningitis.

While the onset is usually fairly acute, it is rarely so sudden as in later life. Practically always the disease is ushered in with a disturbance of the gastro-intestinal tract. The stools are usually loose and greenish or slimy. Vomiting is by no means as constant a symptom as in older children and adults. It was absent in ten of our twenty-five cases. These gastro-intestinal symptoms do not yield to the ordinary methods of treatment. There is practically always an irregular fever. The child is usually irritable and hyperesthetic. There may be convulsions early, but these are not so severe as those that occur later in the disease. Convulsions are not a constant feature. They were present in practically half of our cases in the early stages. The classic signs of meningitis, such as rigidity of neck and the Kernig sign, are usually entirely absent until the case is well advanced. The reflexes at the beginning are normal, as a rule. A bulging fontanel is practically always found. A hemorrhagic rash is very unusual in infants.

On account of the obvious difficulties in diagnosis, we are usually called in consultation when the disease is fairly well advanced. It cannot be too strongly urged that a lumbar puncture should be done early in infants when the diagnosis is indefinite. Netter says: "Think of meningitis and do a lumbar puncture in every infant sick without apparent cause."

Treatment and Course of the Disease

A certain number of cases of epidemic meningitis clear up very readily with the serum treatment. The fatal cases do not often terminate quickly, the course of the disease usually being three weeks or more. It occasionally happens that a patient who has apparently recovered develops hydrocephalus and dies several months after the acute stage has terminated.

In a number of instances in which ventricular puncture was done and the serum administered by that route, the fluid became sterile but the adhesions remained. In only two of this group of cases was ventricular puncture followed by recovery, and it is doubtful whether a real basilar meningitis had developed in these instances. One older child, not in this series, recovered after ventricular puncture. In several instances we have been able to obtain only a small amount of fluid or have had dry taps, and yet serum has been administered by gravity, with complete recovery.

We have followed a very conservative line of treatment. It is our custom to do a daily lumbar puncture, removing as much fluid as possible and injecting serum by gravity. If 20 cc. or more of fluid is obtained, we do not hesitate to inject about 20 cc. of serum, provided it runs in easily and the patient does not show untoward symptoms. We usually inject a smaller amount of serum than we obtain fluid. But if the amount of fluid is very small we may inject somewhat more serum, the quantity depending on the ease with which it runs in and the condition of the patient. It is necessary to exercise more than ordinary care in removing fluid and injecting serum in these young babies. We do not hesitate to perform ventricular or cisternal puncture if there are definite signs of blocking. Certain workers in meningitis have advocated very early ventricular puncture, but the results thus far published have not been such as to encourage us in adopting it.

We continue the injections of serum as long as there are organisms present in the spinal fluid. Even after the fluid becomes sterile, the serum treatment is continued if the clinical conditions seem to indicate it. Lumbar puncture for the relief of pressure—no serum being administered—may be necessary during convalescence.

The general treatment is that of any other acute infection. It is important to disturb the infant as little as possible. It is necessary to keep the child well nourished.

TABLE 2.

DATA ON CASES OF EPIDEMIC MENINGITIS IN INFANCY

Date	Case	Age	Sex*	Intraspinal Injections	Intraventricular Injections	Outcome	Comment	Sequelae
4/ 2/14	1	3 mo.	Not recorded	3	0	Died	Conjunctivitis	
2/ 9/15	2	3 mo.	♀	1	5	Died	Basilar meningitis	
10/28/15	3	3 mo.	♂	3	0	Recovered	....	Deaf
5/19/16†	4	7 wk.	♂	4	0	Died	Parents refused treatment	
6/12/16	5	3 mo.	♂	5	0	Recovered	Macular papular rash	None
7/20/16	6	3 mo.	♂	4	0	Recovered	Arthritis of knee	None
12/24/16	7	3 mo.	♀	5	0	Recovered	....	None
2/ 1/16†	8	2 mo.	♂	2	0	Died	Treatment refused	
5/ 4/17	9	3 mo.	♂	2	1	Died	Basilar meningitis	
5/13/17	10	3 mo	♂	1	1	Recovered	Unable to trace	
6/ 2/17	11	3 mo.	♀	5	0	Died		
8/23/18	12	10 wk.	♂	4	0	Recovered	....	None
3/18/19	13	3 mo.	♂	4	0	Recovered	....	None
4/21/19	14	3 wk.	♂	4	0	Died	Hydrocephalus developed 3 mo. after acute symptoms	
3/ 4/20	15	11 wk.	♀	8	3—	Died	Basilar meningitis	
5/30/21	16	3 wk.	♂	3	17	Died	Basilar meningitis; fluids became sterile	
7/ 5/21	17	3 mo.	♂	0	Several	Died	Basilar meningitis	
11/ 5/21‡	18	3 mo.	♀	0	..	Recovered	....	None
4/20/22	19	3 mo.	♂	8	0	Recovered	....	None
1/ 6/23	20	3 mo.	♂	7	11	Died	Basilar meningitis	
7/23/23	21	3 mo.	♀	Several	Several	Died	Basilar meningitis; left hospital against advice	
1/12/25	22	3 mo.	♂	7	0	Recovered	....	None
4/ 3/26	23	11 wk.	♂	2	0	Died	Hypersensitive to serum	
4/29/26	24	5 wk.	♂	6	0	Recovered	....	None
10/19/26	25	2 mo.	♀	3	2	Recovered	....	None

* In this table, ♂ indicates male and ♀ female.

† Not counted in statistics, as treatment was refused.

‡ One lumbar puncture, no serum; prompt recovery.

MORTALITY

In our group of twenty-five cases, two are not included in the mortality as treatment was refused. Of the remaining twenty-three patients, eleven died, a mortality of 47.8 per cent. This is somewhat higher than our mortality during the first year of life, 46 per cent., and far exceeds our mortality during the second year of life, 29.2 per cent.

REPORT OF CASE

It may be of interest to report in detail the treatment employed in our youngest patient to recover.

D. I., an Italian boy aged 5 weeks, seen April 29, 1926, had become ill ten days before, when the infant was 25 days old. The onset had been sudden with vomiting, severe convulsions, and a temperature of 104 F.

When the baby was examined, the convulsions and vomiting were persisting and the temperature was still 104 F. The fontanel was bulging. The head was retracted. The patellar and pupillary reflexes were normal. Otherwise, the physical examination was negative.

At the first lumbar puncture, 20 cc. of turbid fluid was obtained and 15 cc. of antimeningitis serum administered. April 30, the baby's general condition was improved. Fluid was not obtained on lumbar puncture, but 10 cc. of serum ran in easily by gravity.

May 1, 5 cc. of turbid fluid was obtained, and 10 cc. of serum administered.

May 2, 3 and 4, spinal fluid could not be obtained, but on each occasion 10 cc. of serum ran in easily by gravity.

After the first lumbar puncture the fontanel ceased to bulge and the infant showed progressive improvement. The vomiting and convulsions ceased and the temperature fell to normal, May 4.

The baby has been kept under close observation for eight months, and it has remained well.

This is a case in which many would have resorted to ventricular puncture or cisternal puncture, and would have attributed the recovery to these methods of treatment. This case is by no means an isolated instance of recovery when little or no fluid has been obtained on several occasions, and yet serum has been administered intraspinally with equally good results.

SEQUELAE

The sequelæ were surprisingly few. Of the twelve patients who recovered, one could not be traced. Of the eleven that could be followed up, one is deaf and the other ten have made a complete recovery.

Table 2 gives the more important data in regard to our cases of epidemic meningitis.

For bibliography see the original article.

A CASE OF MENINGITIS CAUSED BY THE MENINGOCOCCUS AND THE PARATYPHOID B. BACILLUS.

Published in The Archives of Pediatrics, 1925, Vol. XLII, pp. 607-609.

Emanuel Appelbaum

The following case, because of its rarity, was deemed worthy of recording in the literature. The patient, an infant boy seven months old, was seen by us for the first time on June 18, 1921. The history of present illness was as follows: Three weeks previously the child was taken suddenly ill with a high fever (105°F.) During the course of the last three weeks the temperature oscillated between 100° and 104°. For the past week the baby had several convulsive seizures and attacks of projectile vomiting. The bowels were constipated. Mentally the child was quite apathetic.

The family and past history were essentially negative.

Physical examination showed the patient to be acutely ill and apathetic. The temperature was 100°; the pulse 120; and the respirations, 20. There was a double internal strabismus. The pupils reacted well to light. The pharynx was slightly congested. The ears were normal. The lungs were clear. The heart, except for the rapid rate of 120, due to the fever, was normal. The abdomen was moderately tympanitic, and the spleen was not felt. There was no cutaneous eruption of any kind, but there was a moderate tâche cérébrâle. The child was not particularly hyperesthetic. The fontanel was not bulging or tense—in fact, there was a depression in this region. There was, however, marked rigidity of the neck with marked retraction of the head. The patellar reflex was normal on the left, but exaggerated on the right side. The abdominal reflexes were present. The Babinski, Oppenheim, Gordon, Schaeffer, and the Chaddock were all negative. No ankle clonus could be elicited. The Kernig sign was positive on both sides, and both the Brudzinski signs were positive. Fundoscopic examination showed a mild optic neuritis in both eyes.

The diagnosis lay between epidemic cerebrospinal and tuberculous meningitis, with many points in favor of the latter.

A lumbar puncture was then performed, and 35 cc. of a cloudy fluid was withdrawn under slightly increased pressure. After the removal of the fluid, 20 c.c. of antimeningococcic serum were introduced. Examination of the fluid showed a great increase in cells, mostly polymorphonuclears, a large amount of albumin and globulin, but no reduction of Fehling's solution. A stained smear showed the presence of Gram-negative diplococci. These organisms were also found on the culture and proved to be meningococci.

June 19:—The fluid obtained by lumbar puncture on this date was distinctly purulent. It showed, however, the presence of a trace of sugar, as there was a slight reduction of Fehling's solution. The meningococci were again found on the smear but not on the culture. Antimeningococcic serum was again given intraspinally.

June 20:—Child seemed a little brighter. Lumbar puncture was performed again, and antimeningococcic serum administered. The fluid was in all respects similar to that of the preceding day.

June 21:—No organisms could be found in the stained smear of the spinal fluid this time, although in all other details this last fluid did not differ from the preceding ones. Antimeningococcic serum was again injected after the removal of the fluid.

June 22:—Lumbar puncture on this day resulted in obtaining 25 c.c. of cloudy fluid which still showed a great increase in cells and contained a large amount of albumin and globulin. The sugar content was, however, definitely increased, as there was a better reduction of Fehling's solution. No organisms were found on smear, but the 24 hour culture yielded a Gram-negative bacillus. A study of the morphological and cultural characteristics, and of the agglutination reaction proved this bacillus to be paratyphoid B.

June 23:—Retraction of the head seemed more pronounced. The spinal fluid was still cloudy, containing many pus cells. The protein and sugar content was the same as the day before. The paratyphoid bacilli were found both on the smear and culture. No meningococci were found. The intraspinal injection of antimeningococcic serum was again repeated.

June 24:—The spinal fluid this time showed only a faint reduction of Fehling's solution, denoting a decided drop in sugar content. Paratyphoid bacilli were present. Antimeningococcic serum was injected intraspinally after removal of the fluid.

June 25:—On this date the spinal fluid was very thick and purulent, contained albumin and globulin in large amount, and did not reduce Fehling's solution. Paratyphoid bacilli were present on smear and culture.

No serum was given this time, but a mixed autogenous vaccine, consisting of both meningococci and paratyphoid B. bacilli, was now administered, intraspinally. A few hours after the vaccine treatment there was a marked reaction, the temperature rising to 105°.

June 26:—By this time the baby's general condition was very bad. A lumbar puncture was done however, and vaccine was again administered intraspinally. The fluid was in all details similar to that of the preceding day.

June 28:—The baby was now stuporous. The temperature was 101°. Only four cc. of purulent fluid were obtained by lumbar puncture this time. Like the preceding fluid, it showed the presence of paratyphoid bacilli. A small amount of vaccine was injected intraspinally.

In spite of the active treatment the child became progressively worse and died on the night of June 28.

COMMENT.

We have here, then, a case of meningitis caused by two different organisms—first, by the meningococcus and followed immediately by the paratyphoid B bacillus. The former organism was recovered from the first three, and the latter from the last six fluids. We have reason to believe that by the use of the anti-meningococcic serum we probably succeeded in controlling the meningococcic infection, but we failed to check the paratyphoid infection by means of the vaccine. It is also probable that the paratyphoid meningitis was the direct cause of death.

MENINGITIS CAUSED BY BACILLI OF THE COLON GROUP

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Vol. CLXII, pp. 740-748.

JOSEPHINE B. NEAL

MENINGITIS caused by organisms of the colon group is comparatively rare. In analyzing more than 1500 cases of meningitis seen by the Meningitis Division of the Research Laboratory up to 1924, only 5 cases were found due to the Bacillus coli in pure culture. A sixth case was due to a mixed infection, one of which was a member of the colon group. There were 2 cases caused by Friedländer's bacillus.

Barron, in a careful study "Meningitis in the Newborn and in Infancy," found that in 42 cases, 39 of which were under three months of age, there were the following classified according to etiology:

TABLE 1.

Bacillus coli	14
Streptococcus and staphylococcus	10
Meningococcus	5
Pneumococcus	4
Tubercle bacillus	3
Bacillus mucosus capsulatus	1
Bacillus influenzæ	1
Bacillus typhosus	1
Bacillus lactis aërogenes	1
Bacillus pyocyaneus	1
Bacillus catarrhalis (?)	1
	42

All except 1 of these cases were collected from the literature. In the earlier cases the diagnosis was often made at necropsy and it seems probable that in many instances the Bacillus coli may have been an invader just before or just after death and not the specific cause of the meningitis. Certainly this distribution by etiology is in marked contrast to that in 50 cases of our own infants during the first three months of life. In our list the diagnosis was made by the examination of the spinal fluid, and the meningococcus was by far the most frequent cause.

TABLE 2.

Meningococcus	24
Streptococcus	11
Tubercle bacillus	5
Pneumococcus	3
Bacillus coli	3
Bacillus influenzæ	2
Micrococcus catarrhalis	1
Streptococcus hemolyticus and Staphylococcus albus	1
	50

The following table shows the cases collected from the literature and also our own cases:

TABLE 3.

CASES OF MENINGITIS CAUSED BY BACILLI OF COLON GROUP

Source.	Year.	Organisms.	Number cases.	Remarks.
Lloyd	1898	Bacillus coli	1	
Concetti	1900	Bacillus coli	4	
Nobecourt and Du Pasquer	1902	Bacillus coli	1	
Kolle and Wassermann	1903	Bacillus coli	13	
Braillon and Merle	1914	Bacillus coli	1	
Milner	1915	Bacillus coli	1	
Michael	1916	Bacillus coli	1	
Frank	1916	Bacillus coli	1	
Barron	1918	Bacillus coli	11	3 additional cases in Kolle and Wassermann.
Sickenga and Munk	1924	Bacillus coli	1	
Meningitis Division	1925	Bacillus coli	6	1 mixed infection.
Total Bacillus coli			42	
Guinon and Simons	1910	Friedländer's bacillus	1	
Bonhoff and Esch	1912	Friedländer's bacillus	1	
Kolle and Wassermann	1913	Friedländer's bacillus	10	
Haig	1919	Friedländer's bacillus	2	
Elias	1924	Friedländer's bacillus	2	
Menetrier and Bertrand-Fontaine	1924	Friedländer's bacillus	1	
Lion and Minvielli	1924	Friedländer's bacillus	1	
Meningitis division	1925	Friedländer's bacillus	2	
Total Friedländer bacillus			20	
Beitzke	1904	Bacillus lactici aërogenes	1	
Kolle and Wassermann	1913	Bacillus lactici aërogenes	2	
Total B. lactici aërogenes			3	
Greenthal	1921	Bacillus acidi lactici	1	
Sherman	1922	Bacillus acidi lactici	1	
Ray	1923	Bacillus acidi lactici	1	
Total B. acidi lactici			3	
Total colon group			68	

As the typhoid group is so closely allied to the colon group it may be mentioned that we have had only one case in which any member of the typhoid group was involved. This was originally a case of meningococcus meningitis but the paratyphoid Bacillus appeared in the fluid during the course of the illness. The case was reported by Appelbaum. A very complete study of typhoid meningitis was made by Bayne-Jones in 1917.

The following table gives certain data in regard to our cases:

TABLE 4.

Year	Case No.	Age	Sex.	Primary focus.	Approximate duration.	Outcome.
				Friedlanders' Bacillus		
1915	38	2½ yrs.	M.	Pneumonia and otitis media	About two weeks with meningitis	Recovery
1920	221	59 yrs.	M.	Otitis media	Six days	Died.
				Bacillus Coli.		
1923	29	2 wks.	M.	Not determined	Twelve days	Died.
1920	360	5 wks.	M.	Not determined	About 8 weeks	Recovered from meningitis developed hydrocephalus died almost a year later.
1923	151	8 mos.	F.	Pyelitis	About 3 weeks	Died.
1916	665	1½ yrs.	M.	Not determined	About 4 weeks	Died.
1919	316	6 yrs.	F.	Infection of orbit	Eight days	Died.

As these cases are comparatively rare a brief description may be of interest.

Case Reports. Case XXXVII.—W. S., a boy aged two years and seven months, was admitted to Willard Parker Hospital March 20, 1915. The onset of the illness on March 19 had been sudden, with a fever of 104° F. He had quickly become stuporous. When examined on March 20 the temperature was 104° F., pulse, 160; respiration, 40. There was a slight stiffness of the neck, a positive Macewen sign, and Kernig sign, no Brudzinski or contralateral reflex. The patellar reflexes were exaggerated, the eyes were normal; there was a discharge from both ears and tenderness around the right mastoid. He was somewhat stuporous. On lumbar puncture 50 cc. of turbid fluid was withdrawn and 25 cc. of antistreptococcus serum was injected, as the meningitis was apparently secondary to the ear involvement. The fluid showed a very great increase in cells, 95 per cent. polymorphonuclears, a moderate increase in the albumin and globulin and a normal sugar content. No definite organisms were found in the smear and it was thought that the culture was contaminated. On March 21, 50 cc. of blood-stained fluid was withdrawn and 20 cc. antistreptococcus serum injected. This fluid showed much the same picture as the first, but a Gram-negative diplobacillus (Friedländer's) was cultured from the fluid, also a diphtheroid bacillus, thought to be a contamination. The patient's general condition was slightly better, but at this time ptosis of the left eyelid appeared, together with dilatation of the left pupil and paralysis of the third nerve on the left side. On March 22, 50 cc. of blood-tinged fluid was obtained. This fluid was sterile as were also the fluids withdrawn on March 22, 24, 25 and 27. These late fluids showed a progressive decrease in the number of cells and in the protein content. The patient's condition also improved. On March 24 an autogenous vaccine was given for the first time in a dose of 250 million. As the spinal fluid had already become sterile, the vaccine cannot be given the credit for the cure of the meningitis. During convalescence from the meningitis a pneumonia with a positive blood culture developed, and an operation on the mastoid was performed. The patient eventually made a complete recovery.

Case CCXXI.—Father S., an Italian priest aged fifty-four years, was seen on June 12, 1920. He had suffered from diabetes and nephritis for several years and from a slight right facial paralysis for some time. Three months before, an acute otitis media had developed. He had been taken ill very suddenly on June 7, with vomiting and chilly sensations. He became unconscious within two hours. There had been no fever for the first two days of his illness. When examined on June 12 he had a temperature of 102° F., pulse. 78; respiration, 46. There was moderate stiffness of the neck and Kernig sign.

The pupils were equal and reacted to light. The patellar reflexes were absent. At times he was delirious and at times stuporous. On lumbar puncture 50 cc. of cloudy yellow fluid was obtained and 20 cc. of antimeningococcus serum injected. The fluid showed an enormous increase in cells, practically all polymorphonuclears, a great increase in the albumin and globulin, and no sugar. In the smear there were numerous short Gram-negative diplobacilli both intracellular and extracellular. A pure culture of Friedländer's bacillus was grown. The patient died on June 13.

Of the cases due to the colon bacillus the second was of unusual interest and will be described last in more detail. The others briefly were as follows:

Case XXIX.—Baby S., aged two weeks, was seen January 30, 1923. The baby had vomited at times since birth and there had been greenish stools. It was difficult to tell when the meningitis had first appeared as meningeal symptoms in infancy are so indefinite. The physician in charge of the case thought it had lasted for about a week. When examined on January 30, the temperature was 100° F., the pulse rapid but regular. The fontanel was bulging, the pupils were equal and reacted to light. The other reflexes were difficult to obtain. As in all infants in the earlier stages of meningitis the stiffness of the neck and Kernig sign were absent. On lumbar puncture 30 cc. of cloudy fluid was obtained and 15 cc. of antimeningococcus serum was injected. The fluid showed an increase in cells, practically all polymorphonuclears, a great increase in albumin and globulin, and an absence of sugar. In the smear there were many short Gram-negative bacilli. A pure culture of colon bacillus was grown. These findings were practically the same in the subsequent fluids. On January 31, 5 cc. of blood-tinged fluid was obtained by lumbar puncture and on February second 0.5 cc. of very purulent fluid. On February 3 and 4 ventricular punctures were done, yielding 45 cc. and 10 cc. respectively of purulent fluid. On February 15, the patient's condition was so serious that no puncture was done. Death occurred on the afternoon of February 15.

Case CLI.—B. T., female infant, aged eight months, was seen on April 23, 1923. The onset of the illness was five days before with fever of 104° F., vomiting, convulsions, cough and irritability. There was a serous discharge from the right ear. There was no stiffness of the neck. The Kernig and Brudzinski signs were negative. The fontanel was not bulging. The pupils were equal and reacted to light. The patellar reflexes were normal. There was marked congestion of the pharynx. The heart and lungs were normal. The spleen was slightly enlarged. The temperature was 101° F. pulse, 120; respiration, 34. As the meningeal symptoms were indefinite no puncture was done. On April 24, as the condition was not improving, a lumbar puncture was performed, 15 cc. of clear fluid being withdrawn. This fluid showed a moderate increase in cells, 90 per cent. mononuclears. The protein and sugar were normal and there were no organisms by smear or culture. It was thought that the condition was one of meningism, probably secondary to the otitis media. The child's condition did not improve and a second puncture was done April 27, yielding 30 cc. of slightly blood-tinged fluid. This fluid was apparently normal except for the blood present. On May 5, a third puncture was done, 20 cc. of clear fluid being withdrawn. This fluid was normal except for a very slight increase in mononuclears and in protein. A week later the child seemed worse and showed more evidence of meningeal involvements. On lumbar puncture May twelfth, 3 cc. of very purulent fluid were withdrawn. This fluid showed a very great increase in cells —polymorphonuclears—and in protein and no sugar. Numerous short Gram-negative bacilli were found in the smear and a pure culture was grown of motile, Gram-negative bacilli which fermented, with the formation of acid and gas, dextrose, mannite, maltose, lactose, dulcite and saccharose. This identified the organism as Bacillus coli communior. Later the patient was catheterized and a pure culture of the same organism was isolated from the urine. We, therefore, concluded that the meningitis was secondary to the pyelitis. Lumbar punctures were done daily until May 18 when it was necessary to do a ventricular puncture as but a few drops of fluid could be obtained from the spine. The ventricular fluid was sterile. On May 19, no fluid could be obtained from the spine but the general condition was somewhat better. On May 21 the child was somewhat more restless and the temperature was 103.5° F. A second ventricular puncture was done yielding 20 cc. of blood-tinged fluid which was sterile. On May 24 a little thick blood-tinged fluid was obtained from the spine. This was also sterile. A catheterized specimen of urine was much less cloudy. The temperature was normal and the general condition seemed much better. This improvement continued until the night of May 26 when convulsions began. A ventricular puncture on May 27 yielded 35 cc. of purulent fluid containing large numbers of organisms. The child died on May 28.

Case DCLXV.—E. D., a male infant aged one and a half years, was first seen on October 22, 1916. There had been a sudden onset, with high fever on October 8. There had been no vomiting. On examination the child was stuporous; the neck was stiff and there was a positive Kernig sign. The Macewen sign was very marked. The patellar reflexes were normal. The temperature was 104° F. On lumbar puncture 40 cc. of turbid fluid was withdrawn and 20 cc. antimeningococcic serum given. This fluid showed a great increase in cells—polymorphonuclears—and in protein and a very low sugar content. In the smear a few Gram-negative "cocci" were seen but the culture was sterile. A second puncture was done on October 23, twenty cc. of fluid being withdrawn and 20 cc. of serum being given. The findings in the second fluid were identical with those in the first. The parents refused further treatment and the case was removed to a hospital where the serum treatment was continued. It was seen by us at the hospital on November 17, when 5 cc. of purulent fluid was obtained by lumbar puncture. This showed numerous short, thick, Gram-negative bacilli in the smear and a pure culture of the colon bacillus. The child died a few days later. It is impossible to say whether this was originally a meningococcus infection or whether the cocci seen in the first two smears were very short bacilli. Organisms in spinal fluid often show pleomorphic forms and we, therefore, feel that we can rely very little on morphology.

Of course, the failure of the organism to grow would be more in favor of the meningococcus, as the colon bacillus grows far more readily.

Case CCCXVI.—M. K., a girl aged six years, was first seen December 10, 1919. She had had measles in May. Following this an abscess had developed behind the right eye necessitating the enucleation of the eyeball. She had complained of pains in the right ear for about a month and had seemed tired and irritable for several days. There had been vomiting for four days. She had been worse on the night of December 9, with fever. When examined she was somewhat irritable, there was moderate stiffness of the neck and a marked Kernig and Macewen sign. The pupil was dilated but reacted to light. The patellar reflexes were absent. There was some tenderness over the right mastoid. The eye socket showed some inflammation. There was profuse sweating. The temperature was 102° F., pulse, 120; respiration, 34. On lumbar puncture 40 cc. of cloudy fluid was withdrawn and 20 cc. of antimeningococcic serum administered. This fluid showed a great increase in cells—polymorphonuclears—and in protein and no sugar. The smear was apparently negative but the culture showed a few colonies of Bacillus coli, which were considered a contamination. Lumbar punctures were done and serum was administered on December 11, 12 and 13. The child's condition improved. The spinal fluids were sterile, became clearer, showed a diminution in the protein and an increase in the sugar. On December 14 and 15 the general condition was not so good and the temperature rose. On December 16 the temperature was 105° F. and the pulse, 160. A lumbar puncture yielded 50 cc. of very purulent fluid from which the colon bacillus was again isolated, this time in great numbers. It seemed probable that a nonspecific action of the serum had produced a temporary control of the infection and an amelioration of the condition. I have seen this same occurrence in other cases of purulent meningitis. The child died on December 17.

Case L. S.—By far the most interesting case in the series was that of L.S. He was born on October 6, 1920. The delivery was normal but he showed signs of collapse and difficulty in breathing for about ten hours. He had slight twitchings for the first few days but these ceased and he seemed normal except for signs of a patent foramen ovale, until the onset of his illness, November 2, 1920. His weight at that time was 10 pounds 7½ ounces. He had weighed 10 pounds 3 ounces at birth. The early symptoms were rather indefinite—sight twitchings, vomiting, a temperature ranging from subnormal to 101.5° F. Early on the morning of November 7, he had a severe convulsion and was seen in the evening of that day. At that time he was in a stuporous condition, the head was thrown back, the fontanel was bulging, the patellar reflexes were not elicited. He was taking nourishment badly and was vomiting profusely. The temperature was 101° F., pulse 160 and poor in quality. A lumbar puncture had been done by another physician earlier in the day and 5 cc. of purulent bloody fluid had been obtained. He had reported that meningococci were present in the smear. A second lumbar puncture was done yielding 3 cc. of purulent, sanguinous fluid and as there were marked pressure symptoms a ventricular puncture was done, 45 cc. of very purulent sanguineous fluid withdraw and 20 cc. antimeningococcic serum injected by gravity. This fluid was uniformly sanguinous and was the most purulent that we have ever received at the laboratory where over 12,000 fluids have been examined. When a test tube full was allowed to stand only a thin layer of clear fluid appeared at the top; over nine-tenths was thick pus. The child's condition was so precarious that it seemed doubtful if it would live until morning. The examination of the fluid showed an enormous increase in polymorphonuclear cells and in protein and an absence of sugar. In the smear there were numerous short, thick, Gram-negative bacilli which grew in pure culture. These organisms fermented lactose and dextrose with production of gas and formed indol, thus proving that it was Bacillus coli communis. On November 8 the condition was slightly improved. The convulsions were less frequent and nourishment was being taken better and retained. On ventricular puncture about 40 cc. of fluid similar to the first was withdrawn and 20 cc. of antimeningococcic serum again given as the organism had not yet been determined. On November 9, the baby was having slight convulsive seizures and the pulse was of a poor quality. A ventricular puncture was done, removing 40 cc. of similar fluid, and 20 cc. normal saline was injected into the ventricle, as it seemed safer to do so since so much fluid had been withdrawn. Ventricular punctures were done daily, with one or two exceptions, until November 28, after which a puncture every other day seemed sufficient. Beginning November 18, several doses of autogenous vaccine were given in the ventricle with the saline, as the fluid showed no signs of clearing and vaccine treatment is often beneficial in Bacillus coli infections. The baby's general condition was gradually improving. On November 12, it cried for the first time. The vomiting was less frequent and the feedings were taken better. The pulse became better in quality and somewhat slower in rate. The temperature was usually between 99° and 100° F. The fluid, however, remained under greatly increased pressure and very purulent, although it became less sanguinous. The amount withdrawn varied from 30 to 60 cc. The ventricles were usually injected with saline at each puncture. The findings in the fluid were the same as at the first puncture, except that the growth of the organism became less profuse. Finally the twenty-fifth fluid, withdrawn December 9, was sterile, as were all the fluids thereafter. On December 11, the fluid was conspicuously clearer and showed the presence of sugar for the first time. On December 15, a lumbar puncture was done and 8 cc. of clear fluid obtained. Another lumbar puncture was done on December 18. On December 20, as there were facial twitchings (these had been present earlier but had been absent for over a week) and vomiting, both ventricles were punctures to make sure that there was no intracranial pressure. From the right ventricles 4 cc. of clear fluid was removed and from the left 1 cc. Lumbar punctures were done also on December 23, 26, 29 and January 5.

The temperature was normal after November 27 and the pulse was usually about 120 and of good quality. The general condition improved very satisfactorily. On December 3 the baby's weight was 9 pounds. He gained steadily thereafter until it reached 12 pounds 6 ounces on February 6. The baby slept well, was playful and intelligent, and seemed entirely normal. The head was slightly enlarged, as was to be expected after the prolonged period of increased pressure. At times the fontanel seemed somewhat tense but this lasted only for a short time and did not occur frequently. Finally, however, about March 10 the head began to enlarge rapidly. On March 13, a lumbar puncture was unsuccessful and 60 c.c. of clear fluid was withdrawn from the ventricle. This fluid was normal except for a slight increase in protein. The relief afforded by the puncture was only temporary. Another ventricular puncture was done March 19 and 60 cc. of clear fluid again removed. A suggestion that the baby be taken to a hospital to try operative measures was not acted upon. The hydrocephalus continued and the baby died about 9 months later.

Children under six months of age, suffering from meningitis are particularly prone to develop a subacute arachnoiditis. This is what probably happened in this case—the inflammation continuing and finally completely cutting off the foramen of Magendie. I have seen a similar occurrence in cases of epidemic meningitis when, after ventricular punctures and the intraventricular administration of serum have rendered the ventricular fluid sterile, one or two lumbar punctures have been successful. But in these cases the complete blocking has taken place in a few days, and the duration of the hydrocephalus has been short, death occurring in at most a few weeks.

Conclusions. 1. Meningitis due to members of the colon group is comparatively rare. In studying over 1500 cases of meningitis only 7 were found due to these organisms. In an eighth case there were several organisms present, one of which belonged to this group.

2. Meningitis in the first three months of life is most often caused by the meningococcus—24 out of 50 cases being due to this organism and only 3 to the Bacillus coli.

For bibliography see the original article.

DIRECT CULTIVATION OF TUBERCLE BACILLI FROM SPINAL FLUIDS. OCCURRENCE OF HUMAN AND BOVINE TYPES

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Ruth Gosling and John Montanus

For some years the Meningitis Division of the Department of Health, New York City, has been interested in determining the relative incidence of human and bovine infection in cases of tuberculous meningitis.

In the past we used guinea-pigs as a means of isolating the organism. The technique was as follows: 5 to 8 cc. of spinal fluid or the emulsified sediment obtained by centrifuging 10 to 15 cc. of fluid was injected subcutaneously into the groin of two guinea-pigs. After four or five weeks, or when definite lesions had developed, the animals were chloroformed, autopsied, and the infected glands removed under aseptic conditions and placed in sterile petri dishes. They were then minced with sterile instruments and a large loopful carefully rubbed over the surfaces of three Dorset's and three Lubenau's egg media slants. The tubes were then sealed with sterile paraffin and incubated at thirty-seven and one-half degrees (37.5°) C. When a good growth had appeared, in about twenty-one to twenty-eight days, they were submitted for typing.

Owing to the fact that the guinea-pigs, because of accidental infections, often died before definite lesions could be demonstrated, it occurred to us that, although spinal fluid contains very few organisms, it might be possible to cultivate these organisms by direct planting upon artificial media.

In this paper we are presenting, first, data to show that tubercle bacilli can be cultivated directly; second, that cultivation is possible even when the number of tubercle bacilli present is so small that they cannot be demonstrated in the direct smear of the centrifuged sediment; and third, a new series of cases to show the relative incidence of human and bovine types in tuberculous meningitis.

The sediments from thirty-four fluids were studied. At first Petroff's beef-bouillon egg medium was used. This contains gentian violet as an inhibiting agent for possible contaminating organisms, but since spinal fluids are seldom contaminated when withdrawn under proper aseptic conditions and collected in sterile containers, we decided to use the same media as we used for our glands, namely, Dorset's and Lubenau's egg media. The technique was as follows: As much spinal fluid as remained (including the fibrin web when possible) after the necessary 15 cc. had been taken to make the diagnostic smear, was certifuged at high speed, in the ordinary conical centrifuge tube, for two hours. All but 0.5 cc. to 1 cc. of the supernatant fluid was poured off and discarded. The sediment was resuspended in the remaining fluid by thorough agitation and scraping of the bottom of the tube with a rather stiff sterile platinum loop. This suspension was removed from the tube with a sterile 1 cc. pipette and distributed over the surface of five slants, two Dorset egg, two Lubenau's and one coagulated blood agar slant. The egg media tubes were sealed with sterile paraffin and incubated at 37.5° C. The coagulated blood agar slant served as a control for contaminating organisms and was also incubated at 37.5° C.

All of our plantings from which the tubercle bacillus grew showed good growth in from twenty-one to twenty-eight days of incubation. These cultures were then submitted for typing to Doctor Grund.

Although rabbit virulence is generally conceded to be the final criterion for determination of types, Park and Krumwiede, with whom Dr. Grund was a collaborator, state in their monograph of 1910, the following: "Culturally one can differentiate practically all viruses with surety. In cases of doubt or lack of sufficient experience in the handling of cultures the intravenous inoculation of 0.01 milligrams will settle the question. Observations of culture and virulence must be done in the early generations." All of our typing was done from the first generation.

Two of the cultures in which there was some doubt as to type were injected into rabbits. These, indicated in the following table, proved to be human. The table gives the results of our thirty-four fluids.

It will be noted that, of the thirty-four sediments planted, twenty-five or 73.3 per cent. yielded positive cultures; six remained sterile, perhaps because of the necessity of using the greater amount of available material for the diagnostic smear, and three showed contaminations in both the egg media and the control shortly after planting. Of the twenty-five which yielded growth of tubercle bacilli, two were from sediments which, after prolonged search, failed to show acid fast organisms in smear, and two showed acid fast granules (Granules of Much).

Only one of these thirty-four stains proved to be bovine.

When we first began planting our fluids directly upon egg media, we thought it of interest, whenever the amount of fluid permitted, to run guinea-pig isolations parallel with the direct method. This was carried out with twelve fluids. Two pigs were used for each fluid. Four sets of pigs died within a week to ten days of inoculation and showed no lesions. The plantings from the sediments from these same fluids yielded tubercle bacilli. This experience suggests that if the amount of fluid permits, the direct cultural method, in addition to the animal inoculation, may be the means of confirming the diagnosis and also the saving for further study of a strain which otherwise might have been lost if animal inoculation alone had been relied upon.

We compared the results obtained by our direct method with those obtained earlier by animal inoculation. A series of thirty-four consecutive fluids was taken, some showing positive smears and some apparently negative and we found that the percentage of positive results was the same for both methods.

The point may be brought up that in the direct cultural method there is the danger of the loss of some particular strain, because of faulty technique or of contamination of the media by some organism in the media itself which does not appear until after prolonged incubation. If guinea-pig glands are used, there is the same danger inasmuch as the same medium is used for the primary isolation of the organism from the gland as from the spinal fluid. Of course if the spinal fluid is contaminated, inoculation into the guinea pig will often result in glands from which the tubercle bacillus may be grown in pure culture, but it has been our experience that spinal fluids withdrawn under proper precautions are rarely contaminated. Possibly the question of time may be one to be considered. We were able by direct method to type

TABLE 1.

RESULTS OF DIRECT INOCULATION OF THIRTY-FOUR SPINAL FLUID SEDIMENTS ON CULTURE MEDIA

	Spinal fluid number	Age of patient	Acid fast organisms in smear	Growth on glycerine egg medium	Growth on water egg medium	Type	Slant control
1	X	Child	+	(Petroff)+	—	Human	—
2	Y	Child	+	(Petroff)+	—	Human	—
3	9128	3 yrs.	+	—	(Petroff)+	Bovine	—
4	9324	8 yrs.	+	+	—	Human	—
5	9337	4 yrs.	+	+	—	Human	—
6	9341	5 yrs.	Acid fast granules	conta	minat	ed	Cont.
7	9361	11 mos.	—	+	—	Human	—
8	9362	27 yrs.	+	+	—	Human	—
9	9370	Child	+	+	—	Human	—
10	9376	Child	+	+	—	Human	—
11	9377	Child	+	+	—	Human	—
12	9380	Unknown	+	+	slight growth	Human	—
13	9381	5 yrs.	+	+	—	Human	—
14	9402	3½ yrs.	+	+	—	Human	—
15	9405	2 yrs.	+	+	slight growth	Human	—
16	9412	18 mos.	+	—	—	—	—
17	9414	3 yrs.	+	—	—	—	—
18	9425	2 yrs.	+	+	slight growth	Human	—
19	9434	3 yrs.	+	+	slight growth	Human	—
20	9435	11 mos.	+	+	slight growth	Human	—
21	9436	23 yrs.	—	+	slight growth	Human	—
22	9448	22 mos.	+	—	—	—	—
23	9460	26 yrs.	Acid fast granules	—	—	—	—
24	Z	Child	+	+	—	Human	—
25	9467	14 mos.	+	+	—	Human	—
26	9470	10 mos.	+	+	—	Human	—
27	9590	2 yrs.	+	contaminated		—	—
28	9618	20 yrs.	+	—	—	—	—
29	9563	5 yrs.	+	—	—	—	—
30	9654	1¼ yrs.	+	contaminated		—	B. subtilis
31	9688	9 yrs.	+	—	slight growth	Human	—
32	9704	10 mos.	+	—	slight growth	Human	—
33	9156	5½ yrs.	+	(Petroff)+	—	Human	—
34	9164	Unknown	+	(Petroff)+	—	Human	—

Results:—Human Bovine
24 1

cultures in twenty-eight days at the most, whereas if guinea-pigs had been used, at least eight weeks would have been necessary for the typing.

As we have indicated in the beginning of this paper, the work of typing was first carried on by guinea-pig inoculation. We isolated by this method twenty-three strains. The results are shown in Table II.

TABLE II.

TYPES OF TUBERCLE BACILLI FOUND IN SPINAL FLUIDS BY GUINEA-PIG INOCULATION

	Fluid Number	Age of Patient	Type
1	7650	3 years	Human
2	7882	8 years	Human
3	7945	2½ years	Human
4	7962	Unknown	Human
5	8136	14 years	Human
6	8200	4 months	Human
7	8310	5 years	Human
8	8318	18 years	Human
9	8477	4 years	Human
10	8499	5 years	Human
11	8503	2 years	Bovine
12	8528	8 years	Bovine
13	8617	8 years	Human
14	8622	Unknown	Human
15	8656	41 years	Human
16	8604	9 years	Human
17	8677	8 months	Human
18	8851	1 year	Human
19	8880	3½ years	Human
20	9007	3 years	Human
21	9118	1 year	Human
22	9305	10 months	Human
23	White	Unknown	Human
	Results	Human 21	Bovine 2

Two of the above twenty-three strains isolated from the guinea pig were bovine. One of the twenty-five isolated by the direct method was bovine. Both of the cases from which the bovine type was isolated by animal passage

TABLE III.

SUMMARY OF CASES OF TUBERCULOUS MENINGITIS CLASSIFIED AS TO TYPE

Reference	Human	Bovine	Total	Percentage of human types
Park & Krumwiede	123	16	139	88.5
Novick	45	3	48	93.7
Gordon & Brown	4	0	4	100
Gosling & Montanus	45	3	48	93.7
Total	217	22	239	90.8%

were residents of New York City. In one case, 9128, only a very indefinite history of the feeding could be obtained from the mother. The child was a breast-fed infant and later given Grade A milk. It could not be ascertained whether or not the milk was Grade A Raw or Pasteurized. The child at the time of the meningitis was on a general diet.

Case 8503 was breast fed until six months. It was then given Grade B Pasteurized milk which the mother said she "pasteurized" again but did not boil. It was never given what the mother called raw "store" milk.

Case 8528, isolated by direct culture, was an out-of-town case, and no history of the feeding could be obtained.

Thus out of a total of forty-eight viruses, three or 6.3 per cent. were of the bovine and forty-five or 93.7 per cent. were of human origin.

Table III is a summary of the cases in the literature as to the relative incidence of the human and bovine types in tuberculous meningitis. This table includes our own findings.

SUMMARY

We have shown that in a fair percentage of spinal fluids from cases of tuberculous meningitis, in which the organisms were demonstrated microscopically, it is possible to grow the tubercle bacillus in pure culture by direct planting of the sediment upon suitable egg media.

The fact suggests itself that direct planting of the sediment from cases of suspected tuberculous meningitis, where no organisms can be shown in smear, may have some value as a diagnostic procedure and further, that when guinea-pigs are used for the isolation of strains of the tubercle bacillus from spinal fluids, especially when conditions are adverse to the proper care of the animals, due to overcrowding and incidence of epizoötic diseases during the winter months, the planting of some of the sediment directly upon egg media may be the means of saving some strains for further study.

In our series of forty-eight strains of tubercle bacilli isolated from spinal fluids from cases of tuberculous meningitis, three or 6.3 per cent. proved to be of bovine origin.

For bibliography see the original article.

STREPTOTHRIX MENINGITIS

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Ten Eyck Elmendorf and Josephine B. Neal

The classification of the higher bacteria to which the streptothrix group belongs presents many difficulties, and no attempt will be made in this paper to undertake a discussion of them.

The infections in man reported as being due to the streptothrix group are comparatively rare. The lung is apparently the chief site of infection but metastatic abscesses of the brain and cord are occasionally reported. Cases of true meningitis not secondary to brain abscesses are apparently much less common.

In a study of streptothrix meningitis published by Neuman and Rabinowitsch-Kempner, in 1922, it is stated that they could collect from the literature but three well established cases of streptothrix meningitis. However, they accepted only those cases that were confirmed by autopsy. The cases they report are as follows: 1. A case reported by Sternberg in 1910, a twelve-year old boy. The meningitis was secondary to a very chronic otitis media. 2. A case reported by Weisner in discussing Sternberg's case. The meningitis was secondary to an acute otitis media. 3. A case reported by von Eicken and Huntemüller, in 1919—an 18-year-old boy, the meningitis being secondary to a chronic otitis media.

Their own case was that of a child three years of age. This meningitis also was secondary to an otitis media. The duration of the illness was only three days. Two lumbar punctures were done. The spinal fluid was purulent and showed the streptothrix in pure culture.

They also refer to a case described by Naunyn, in 1891, in which there was a streptothrix pyemia in the course of which a streptothrix meningitis developed.

A study of literature, however, reveals other cases that apparently must be included in the number of cases of streptothrix meningitis.

Horn, in 1913, reported a case in a man of thirty-five who had suffered for many years from otitis media. He developed symptoms of meningitis and also ocular symptoms indicating sinus infection. It is stated that the spinal fluid was negative. The autopsy revealed purulent meningitis, sinus thrombosis and otitis media. The streptothrix was isolated from the purulent material.

In the Italian literature there are reports of 10 cases of streptothrix meningitis, the diagnosis usually being made by cultures from the spinal fluid. The first case was reported by Rutelli in 1915,—a child six months old. Three lumbar punctures were done. The outcome of the case is unknown as the child was taken to the hospital. In 1916, Rudoni reported seven cases of streptothrix meningitis that developed over a short period during a small epidemic of meningococcic meningitis. In five instances meningococci were found earlier in the spinal fluid and the streptothrix appeared later. In two cases the streptothrix was the only organism found. All the cases resulted fatally. This unusually large number of cases of a rare infection occurring within a period of two months makes one wonder what the source of infection could have been. Such conjectures are usually futile, however, and it seems best to refrain from making them. In 1918

Kharina-Marinucci reported a fatal case in a child four months old. In 1922 Fonzo reported a fatal case in a child 12 months old. This makes a total of sixteen cases of meningitis due to the streptothrix, if we include Naunyn's case which Neuman and Rabinowitsch-Kempner did not include. No attempt has been made to collect the cases of brain abscess either with or without meningitis, but from references to them they are apparently more common than are cases of pure meningitis. Of course as autopsies were not performed in all the cases that we have listed it is possible that a brain abscess may have been present in some of them. However, there was nothing in the clinical histories to indicate that such was the case.

The case that we are reporting gave the following history:—Philip E., 12 years old, had been a blue baby at birth. A heart lesion had been diagnosed during the first year. He had always been extremely delicate. Up to five years of age he had frequent convulsions. His cyanosis was variable in degree but was never absent and he was often prostrated by indispositions that would not have affected a normal child. However, he had adapted himself very well to his physical handicap and had lived a comfortable life. On December 1, 1922, on his return from a visit to Virginia, he complained of headache. On December 2, after going on a shopping trip, he again complained of headache. Neither of these attacks lasted long and they were attributed to the train trip and the shopping. On December 4 he again suffered from headache and on December 5 was seen by his family physician. No cause for the headache could be found. On December 6 he stayed indoors and was in bed part of the time. No definite diagnosis was made. During the next few days he ran a low, irregular fever and vomited once. He was again examined on December 11 and a blood count was done which was reported as follows: Red blood cells, 6,600.000; hemoglobin, 135 per cent.; color index, 99; white blood cells, 10,500; polymorphonuclears, 74 per cent.; small lymphocytes, 24 per cent.; large mononuclears, 2 per cent.; no eosinophiles or basophiles. The urine was also examined and was highly acid but otherwise normal. On the night of December 12 he suddenly became worse. Convulsions developed. On the evening of December 13 he was seen in consultation. He was lying in bed on his left side. His color was quite cyanotic. The face was drawn and there was some flattening on the left side. The tongue was tremulous and could not be protruded. The tonsils were over medium size, very ragged and red. The temperature was 100°F. The right pupil was widely dilated and reacted sluggishly to light. The pulse was fairly regular and full. The rate was 120. The heart was apparently not enlarged. There was a marked systolic and diastolic murmur at the apex; the second pulmonic was accentuated. The respiration was about 60. The examination of the lungs showed a slight general dullness on percussion and scattered moist rales, more numerous at the bases.

There was a marked general hypertonicity of the muscles. The neck was very rigid and so also were the muscles of the abdomen. The knees were drawn up, and the patellar reflexes could not be elicited. The Kernig sign was very marked. A more complete examination was impossible on account of the objection of the mother who was holding the child in her arms.

At about 10 o'clock that evening, when he was seen for the purpose of doing a lumbar puncture, the picture was about the same. At that time there were marked convulsive twitchings of all the muscles. Both pupils were widely dilated and did not react to light. The patellar reflexes were found to be exaggerated. There was a Babinski and an ankle clonus. The pulse at that time was quite irregular, both in rate and in rhythm. A lumbar puncture was done and revealed cloudy fluid under greatly increased pressure. 35 cc. of fluid were withdrawn and 20 cc. of antimeningitis serum injected by gravity. With the aid of a small dose of morphine he passed a fairly comfortable night. On the 14th the condition was about the same. The muscular twitchings were less marked, however. A second puncture was done, no serum being given as the examination of the first fluid showed that the case was not one of meningococcic meningitis. During that night he became much worse and frequent cardiac stimulations were necessary. He died on the afternoon of the 15th.

Examinations of both fluids were practically identical. The fluids were yellowish in tinge and extremely purulent. There was an enormous increase in cells—98 per cent. polymorphonuclears. The albumin and globulin were very greatly increased showing a + + + + massive reaction. The glucose was practically absent as shown by the negative reduction of Fehling's, though the heavy albumin content may have masked the reaction to some extent. The microscopical examination showed numerous microorganisms that appeared to be small gramamphophilic cocci and bacilli. The cultures were negative aerobically on chocolate agar, blood plates, plain agar and broth but anaerobic cultures in a sealed serum broth tube revealed organisms of the same type as those seen in the smear. Not recognizing this organism it was given to Dr. Anna W. Williams who classified it tentatively as belonging to the actinomyces-nocardia-streptothrix group, because of its thread-like forms with swollen ends and its ability to break up into extremely irregular members ranging from minute globoid bodies to larger rounded or oval forms.

After studying the organisms Dr. Williams stated "that the descriptions of most organisms called by any one of these names have been very meager and therefore the classification of the group as a whole is decidedly unsatisfactory. In the first place the name streptothrix for members of this group has been given up by most classifiers because it had already been used for a certain mould. Wright, who wrote the monograph in Osler's Modern Medicine on Actinomyces and similar organisms, revived the term nocardia for those forms that grow freely aerobically, do not form swollen ends in lesions, are Gram-positive and more or less acid fast. He reserved the term actinomyces for strains that produce swollen ends in lesions, grow with difficulty and more or less anaerobically in cultures and are gramamphophilic. In the recent classification recommended by the Society of American Bacteriologists, the term nocardia is dropped and all of the forms including this genus are placed under the genus actinomyces. This genus is placed in the family actinomycetaceae. In this family are included three other genera —actinobacillus, leptotrichia and erysipelothrix. The organism under discussion continued in successive cultures to be strictly anaerobic. On lactose agar it produced abundant branches. It therefore is classified according to the old classification as a streptothrix and according to the latest classification as belonging to the actinomyces."

For bibliography see the original article.

TORULA MENINGITIS

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Louis L. Shapiro* and Josephine B. Neal

The classification of pathogenic yeasts has been the subject of much discussion among bacteriologists. As a result, the nomenclature has been greatly confused. In recent years it has been shown that one of these forms, *Torula,* has a special predilection for the central nervous system and the lungs. The outstanding study of this organism and its rôle in producing infection has been made by Stoddard and Cutler.

Earlier work was done by Türck and von Hansemann in Germany, and valuable contributions have also been made by Brewer and Wood, Pierson, Rusk, Evans, Freeman and Weidman, Sheppe and Bettin, in this country. These workers have reported thirteen cases that may be considered due to the torula, although the cultural characteristics have not always been entirely identical. Sheppe, in a personal communication, reports another case of meningeal involvement.

This makes, with our own, a total of fifteen cases, of which thirteen involved the central nervous system, one the lung and one the muscles of the back and the vertebral column. Of these thirteen cases of involvement of the central nervous system only five were diagnosed during life by the examination of the spinal fluid. In all cases except our own the diagnosis was made only a comparatively short time before death.

Report of a Case

History.—A boy aged 16. born in New York, had spent most of his life in New York and New Jersey. He was 6 feet and 1 inch in height and was well developed but slender. He was attending a private school. He had always been well, and there was nothing of moment in his past history until February, 1923, when he had complained of left frontal headache, weakness and sleepiness, which persisted for about one week. At this time he did not appear to be suffering from any acute or chronic disease. His mentality was normal, and physical examination, especially of the nervous system, eyes, ears, nose and sinuses was negative.

Roentgen-ray examination of the head was negative. The temperature, pulse and respiration were normal. Blood pressure was: systolic, 120, diastolic, 68; homoglobin (Dare) 95 per cent.; urine negative. Suspecting encephalitis, he was kept at home another week, at the end of which time he felt so well that he returned to school.

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TABLE 1.

COLLECTED CASES OF TORULA INFECTION IN MAN (AUTHENTIC CASES).

	No. of Cases	Sex	Age	Race	System Chiefly Involved	Result	When Diagnosis Was Made	Duration of Disease
1. Brewer and Wood	1	M	20	Russian	Muscles and vertebral column	Recovered	Organisms recovered	5 months
2. Pierson	1	M	57	American, California	Central nervous system	Fatal	Post-mortem	6 weeks
3. Stoddard and Cutler	1	F	42	American, Florida	Central nervous system	Fatal	Post-mortem	14 weeks
4. Stoddard and Cutler	1	M	39	American, Massachusetts	Central nervous system	Fatal	Post-mortem	12 weeks
5. Rusk	1	M	57	German, California	Central nervous system	Fatal	Post-mortem	10 months? complicated by syphilis
6. Rusk	1	..	..	German, California	Central nervous system	Fatal	Post-mortem	1 month
7. Türck	1	F	43	German	Central nervous system	Fatal	Post-mortem	6 weeks
8. Von Hansemann .	1	M	18	German	Central nervous system	Fatal	Post-mortem	?
9. Evans	1	M	13	Mexican, California	Central nervous system	Fatal	Ante-mortem	5 weeks
10. Evans	1	F	20	Mexican, California	Central nervous system	Fatal	Ante-mortem	9 weeks
11. Freeman and Weidman	1	M	39	American, Pennsylvania	Central nervous system	Fatal	Ante-mortem	4 months
12. Sheppe	1	M	48	American, Virginia	Lungs	Fatal	Post-mortem	2 months
13. Sheppe (personal communication)	1	M	..	American	Central nervous system	Fatal	Ante-mortem	Few days ?
14. Bettin	1	F	40	American	Central nervous system	Fatal	Ante-mortem	5 weeks
15. Shapiro and Neal.	1	M	16	American, New York	Central nervous system	Fatal	Ante-mortem	7 months

Course of Illness.—He was well until May 15 when, after eating a large quantity of chocolate and pie, he suddenly developed a similar left frontal headache with vomiting, weakness and dizziness. These symptoms persisting for several days, he returned to New York, and was again seen on May 19. Not responding to symptomatic treatment, he was sent to a sanatorium on May 23. Physical examination at this time was negative, except for a loss of 15 pounds (6.8 kg.) in weight since February. Mentally, he was normal until the evening of May 25, when he suddenly became delirious. This lasted but a few hours. Neurologic examination at this time showed exaggeration of both patellar reflexes only. On May 27 the patient complained for the first time of pain in the back of the head and neck, slight disturbance of vision and photophobia. From then until May 29 evidence rapidly developed that we were dealing with an acute infection of the central nervous system, particularly of the meninges. He showed definite rigidity of the neck. The eyes were painful on extreme movement to the left, the pupils were equal in size and reacted normally to light and accommodation. The fundi showed beginning choked disc slightly more developed on the left. There was no definite evidence of cranial nerve involvement. The abdominal and cremasteric reflexes were diminished, especially the right. Both knee reflexes were markedly diminished. The bilateral Kernig sign was positive. Mentally, he was clear but somewhat irritable. The temperature was 101 F., pulse, 52; respiration, 20 and regular. The temperature and pulse previous to this time had always been normal. A cell count revealed: red blood cells, 5,640,000; hemoglobin, 89 per cent. (Dare); white blood cells, 14,400; polymorphonuclears, 80 per cent., and lymphocytes, 19 per cent.

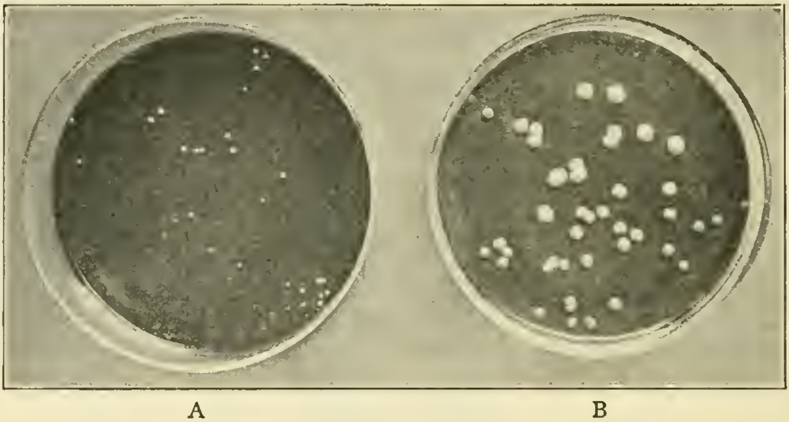

Fig. 1.—*A*, forty-eight hour culture of Torula on glucose veal agar plate; *B*, seven day culture. (Reprinted by courtesy of Archives of Neurology and Psychiatry.)

Lumbar puncture was performed for the first time on May 29. The fluid was found to be under very high tension, and slightly hazy in appearance. There was no growth in the culture of the fluid the first eighteen hours, but distinct yeast colonies were apparent on the following morning, May 31, thereby establishing the diagnosis of a yeast infection of the central nervous system. Lumbar punctures were subsequently made daily until near the end, and the torula organism was recovered from the fluid by culture and observed in smears.

From May 29 until June 18 there was evidence of rapid progress of the disease, with symptoms of increasing intracranial pressure. The patient was frequently delirious and lost weight rapidly. He complained of photophobia and double vision. Rigidity of the neck persisted. The pupils were moderately dilated and were sluggish to light. Distinct weakness of the right and left external recti developed. The fundi showed increasing papilledema, being from 2 to 3 diopters on June 2, and 4 or 5 diopters on June 7. The abdominal and cremasteric reflexes were not elicited. The deep reflexes were generally diminished. A bilateral Kernig sign was present, but no Babinski sign. The temperature was irregular, varying from 98.6 to 103.8 F. The pulse was always slow in comparison with the temperature, ranging from 60 to 100 and averaging about 75. Respiration was normal. On the morning of June 8, the patient went into a deep stupor. Toward evening, he gradually roused after another lumbar puncture and tube feeding, and was rational again the next morning. Tube feeding was necessary from this time on. This was done every six hours, and continued until about the middle of August. In this way, the caloric intake for twenty-four hours was increased from less than 800 to more than 5,000. Up to this stage one lumbar puncture daily was sufficient to relieve intracranial pressure. Now, however, a puncture would afford relief for only about ten hours, when the patient would again become restless, irrational, noisy and cry out as if in great pain. Punctures therefore were made twice a day until June 19, when one puncture again gave sufficient relief.

Tube feeding was discontinued about the middle of August, from which time he took nourishment well, averaging about 5,000 calories in twenty-four hours. He gained considerably in weight and appeared to be well nourished. He was quiet and seemed comfortable most of the time. Mentally, he was for the most part lethargic, confused and disoriented. Frequently, there were periods lasting several days or weeks when at times he seemed quite rational, would talk coherently when spoken to, would recognize people, and appeared to be interested in what was going on about him. He rarely complained of headache and vomited only three times. Weakness of the right and left external recti persisted throughout. The fundi improved considerably, showing an elevation of 1 diopter in the right disk and 1.5 diopters in the left. The margins appeared fairly distinct, and the veins were less tortuous and full. No further cranial nerve involvement appeared. The abdominal and other superficial and deep reflexes remained absent throughout. Kernig's sign was always present. No gross sensory or motor changes were obtained. The temperature was always irregular, showing a morning fall and evening rise. From the middle of June to early in July it varied from 101 to 103.6 F., occasionally reaching 104 and once 105 degrees. During the remainder of July it varied from 100 to 102 F., except for a brief interval. During August and September it varied most of the time between 99 and 101 F., occasionally reaching 102 degrees during September.

The pulse gradually increased in rate, varying during the last half of June between 100 and 120. During July, it ranged between 110 and 130. During August and September, it varied between 130 and 150, averaging about 140. The respirations were always normal and regular. During this entire period the lungs were clear and the heart was normal except for the tachycardia. The blood pressure varied from time to time between 90 and 144 systolic.

During the last week in September scattered, moist râles were detected at both bases posteriorly. On October 1 the patient again became stuporous.

The lungs showed impaired resonance with small areas of distant, high pitched breathing. Pulmonary edema developed on October 8 and the patient died on the evening of October 11 of respiratory failure.

Peculiar pressure sores developed over both hip joints, the inner side of both knee joints and both heels during the last month of the disease. They began as large violet-blue blebs, rapidly breaking down, becoming excavated, but with firm indurated margins. Cultures taken from these areas were negative for torula organisms.

Treatment.—As the smear from the first spinal fluid withdrawn showed forms suggesting gram-negative cocci, antimeningococcic serum in 20 cc. doses was given intraspinally the following four days. When the diagnosis of yeast infection was definitely established, it was decided to give iodids because of their action on other yeast infections, especially the oidiomyces. Sodium iodid, 15½ grains (1gm.) was therefore given intraspinally every other day, beginning June 3, until July 19. One hundred grains (6.5 gm.) of sodium iodid was also given daily internally, beginning May 31, and increased to 400 grains (26 gm.) daily from August 1 to the end of September. The iodids were given again intraspinally every other day during August. The patient tolerated the iodids very well, and at no time showed

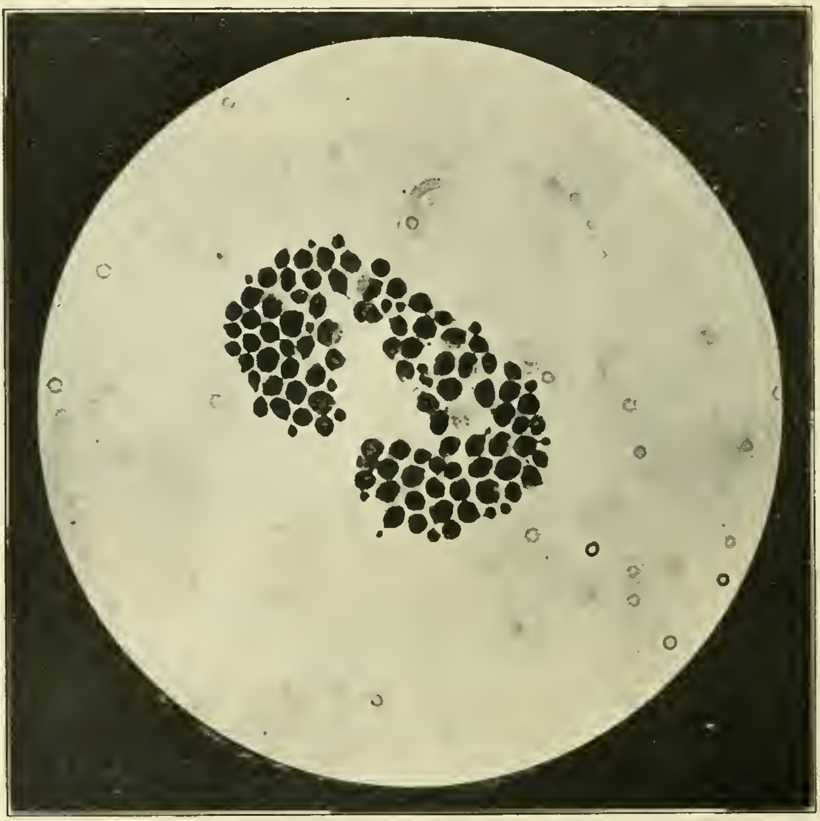

Fig. 2.—Smear of fifteen day culture of *Torula;* budding forms.

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symptoms of iodism. Because of the favorable reports from the use of monilia vaccine in tropical sprue, a vaccine was made from the torula organisms and given twice weekly, beginning June 6 with 500,000, increasing 500,000 with each dose. This was continued throughout the entire course of the disease. Beginning August 25, vaccine prepared by the Dreyer method was used. The patient never showed any local or general reaction.

On July 21, 24 and 26, a colloidal silver preparation was given intraspinally. Each administration was followed in a few hours by a thermic reaction, the temperature rising to 103 F., and gradually declining to 99 F. in forty-eight hours. This was tried on account of a great increase in the number of colonies in cultures. After the first injection there was also a severe general reaction. As there was no evidence of improvement in the patient's condition or of effect on the growth of the organisms, the colloidal silver preparation was discontinued after the third dose.

Serum obtained from rabbits immunized by intravenous injection of the torula was given intraspinally on September 5, 8 and 13, the first dose being 5 cc., the second 3 cc., and the third 5 cc. The third dose was followed by a severe general reaction, with a temperature of 104 F., pulse 170 and respiration 30, so that it seemed inadvisable to continue giving the serum.

Arsenic was given internally in the form of liquor potassii arsenitis (Fowler's solution), 15 minims (1 cc.) daily during the month of August.

A high caloric diet averaging about 5,000 calories in twenty-four hours, well proportioned in proteins, carbohydrates, fats, fruit juices and salts, was given and well tolerated throughout the entire course of the disease. As mentioned previously, tube feeding was resorted to for a period of about two months.

Clinical Pathology.—The Wassermann test was negative on the blood and the spinal fluid. Blood cultures taken June 7 and 29, August 21, September 23 and October 5 were all negative for *Torula*. Complete blood counts were made every ten days throughout the course of the disease. The average count for June was: hemoglobin, 80 per cent. (Dare); red blood cells, 4,840,000; differential count normal; color index, 0.83; white blood cells, 12,400; polymorphonuclear neutrophils, 83 per cent.; lymphocytes, 13 per cent.; large mononuclears, 4 per cent.; eosinophils, none.

On June 8, when the patient was in deep stupor, the white blood cells were 28,000 with 94 per cent. polymorphonuclears. The average counts for July, August and September were about the same. On October 10, the examination showed for the first time qualitative changes indicative of anemia.

The blood chemistry on June 11 was within normal ranges. The findings were; nonprotein nitrogen, 34 mg. per 100 cc. of blood; urea nitrogen, 15 mg. per 100 cc. of blood; uric acid, 2.5 mg. per 100 cc. of blood; blood creatin, 1.5 mg. per 100 cc. of blood; blood sugar, 100 mg. per 100 cc. of blood; whole blood chlorid 363 mg. per 100 cc. of blood; alkali reserve, 70 per cent.

On August 16 an agglutination was made with the torula and the patient's serum, undiluted, and in dilutions of 1:10, 1:50, 1:100 and 1:500. There was no agglutination, although the patient had been receiving vaccine for more than two months. Agglutinations in the same dilutions were made with horse and sheep serum. It was hoped that one of these animals would show some natural immunity to the torula, but none was found.

Throat cultures and cultures of the sputum were negative for torula organisms.

Urine analyses were made practically daily throughout the disease. The average analysis was: specific gravity, 1,023; albumin, trace, with few medium to large finely and coarsely granular and hyaline casts; sugar, acetone and diacetic acid, none.

Cultures of the urine from time to time were negative for *Torula* organisms. The patient had a urethral discharge during September. Cultures from this were negative for *Torula* organisms and gonococci.

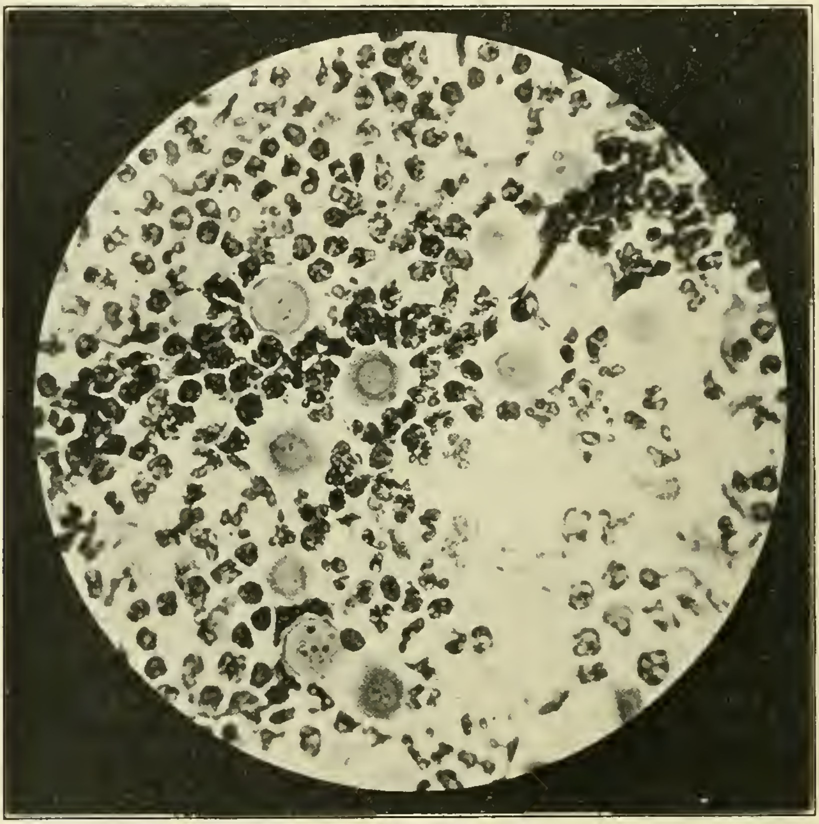

Fig. 3.—Smear of ninth spinal fluid, showing forms of various sizes. Rings of cytoplasm vary in width. Nuclear material is granular in some forms.

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Spinal Fluid Examinations.—The first lumbar puncture was performed on May 29, 1923. The fluid was under greatly increased pressure and was slightly hazy and showed 300 cells per cubic millimeter. Of course, many of these were yeast cells. The other cells were about 90 per cent. mononuclears. Smears were stained both by the Ziehl-Neelson stain and by the Gram method. No tubercle bacilli were found in the Ziehl-Neelson stain, but there were many large heavily stained cells which were later identified as yeast cells. In the smear stained by the Gram method there were seen degenerated cells, with a suggestion of intracellular gram-negative cocci. There was a moderate increase (+ +) in albumin and globulin in the fluid, and the sugar-content as shown by the reduction of Fehling's solution was markedly diminished (+).

The second lumbar puncture, made May 30, revealed 45 cc. of hazy fluid, with practically the same findings as in the first, except that the percentage of polymorphonuclear cells was considerably higher.

On May 31 there was a heavy growth of yeast on the forty-hour culture from the first fluid and a light growth on the twenty-four hour culture from the second fluid. The smears and cultures were submitted to Dr. Anna W. Williams, Assistant Director of the research laboratory.

Daily lumbar punctures were performed until June 8. At each puncture from 30 to 60 cc. of fluid were withdrawn. The chemistry of the fluid remained about the same, but the type of cells was apparently modified somewhat by the administration of serum, as fluids withdrawn after serum was given usually showed a high percentage (80 to 90 per cent.) of polymorphonuclears. A Wassermann test on the second fluid was negative. The *p*H of a number of fluids was ascertained. It ranged from 7.5 to 7.8, which is within the normal limits for spinal fluid and indicates that the torula was not rendering the fluid acid. In the first four fluids the yeast cells were large and stained heavily. The structure was not well shown. They stained best by the Ziehl-Neelson method. With the Gram stain they were amphophilic and less easily distinguishable from the cells of the spinal fluid. After the fourth puncture, the structure of the torula was better shown. The organisms varied greatly in size and appearance. In some, the margin was sharply defined; in others, the outline was indistinct and fuzzy. In some, the greater part of the cell was made up of a densely staining nuclear-like material surrounded by cytoplasm of varying widths. In others, this densely staining nuclear substance was relatively small and surrounded by a wide clear cytoplasm. At times, the center of the cell was apparently broken up into granules, which often took a reddish stain. Budding forms were seen on practically all types of cells. Small forms with practically no cytoplasm might have been mistaken for small lymphocytes if buds had not been seen. The cytoplasm was apparently of a gelatinous nature, as shown by the fuzzy halo. This gelatinous character may also account for the fact that the yeast cells, especially those with a small nucleus and a wide cytoplasm, were often almost completely surrounded by lymphocytes. The gelatinous character of the cytoplasm or capsule has also been noted by Sheppe, who observed that in contaminated cultures the yeast cells were often surrounded by other organisms. In all of these early cultures there was a luxuriant growth in from twenty-four to thirty-six hours.

From June 8 to June 18 inclusive, two punctures were made daily for the relief of pressure. From 40 to 60 cc. of fluid was usually withdrawn at each puncture. These fluids yielded practically the same findings as before. On June 15 the fluids became somewhat clearer, with a corresponding decrease in tissue cells—although they remained greatly increased above the normal. The relative proportion of mononuclears and polymorphonuclears often changed for no apparent reason. The number of yeast cells varied from time to time. On June 16 the culture began to be less luxuriant, and from that time the number of colonies gradually decreased in number and did not appear until forty-eight hours had elapsed. Beginning with June 19, daily punctures were made again. The character of the fluid showed little change, but the colonies continued to diminish in number, and on June 21 did not appear until after seventy-two hours had elapsed. The number of colonies was small, even on plates inoculated with centrifuged sediment. For some time plates had been made with both the centrifuged and uncentrifuged fluid to estimate as well as possible the number of yeast cells. It was noted that there was little relation between the number of colonies and the number of yeast cells seen in the smear. Often when only two or three yeast cells could be found in the smear there was an abundant growth, and we therefore concluded that it was impossible to differentiate the small form of the torula from the lymphocyte. This decrease in the number of colonies and their rate of growth continued until July 16, when the cultures again began to show in forty-eight hours and the number of colonies began to increase rapidly. On August 1 the colonies grew in twenty-four hours. From this time the colonies varied in rate of growth and in number, but they were, on the whole, far less numerous than during the last two weeks of July. The cultures continued positive until September 26, although the number of colonies was small. The cultures on that date were negative, as were also the succeeding five cultures. The character of the fluid remained much the same until August 22, when the fluid became slightly blood-tinged. On August 26 the fluid was yellowish, with no free blood present. This condition continued for some days, when the fluid again became slightly blood-tinged, and thereafter

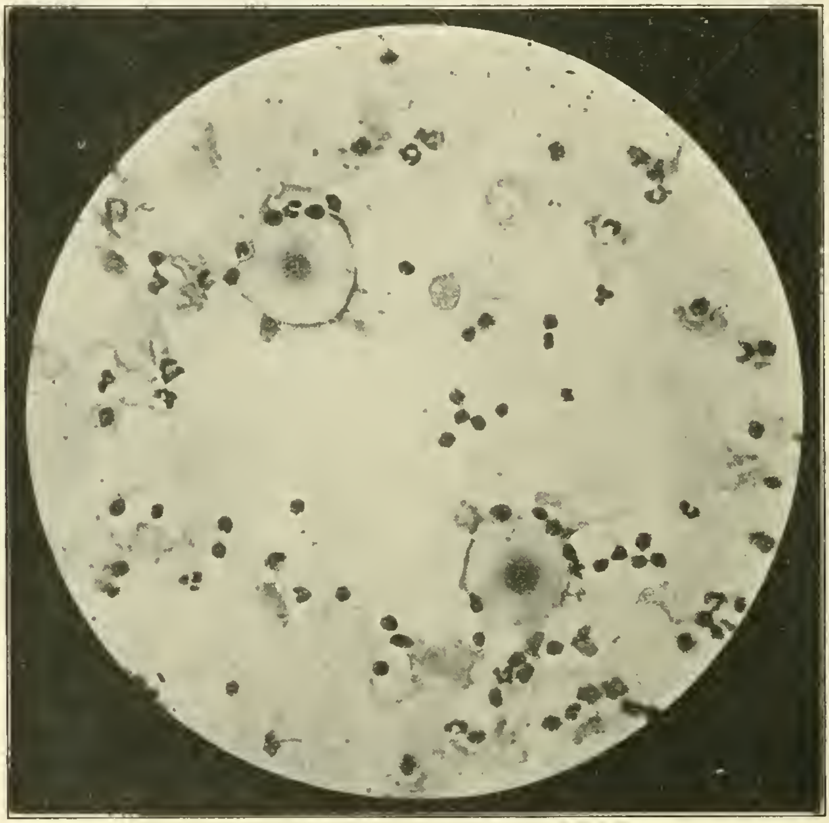

Fig. 4—Smear of twenty-fourth spinal fluid showing *Torula* surrounded by lymphocytes. (Reprinted by courtesy of Archives of Neurology and Psychiatry.)

was either blood-tinged or yellowish until the end. The amount of blood present apparently was not sufficient to affect greatly the protein content, since the quantity of albumin and globulin gradually decreased from a moderate (+ +) to a slight (+) amount by the end of August. The amount of sugar gradually increased and was apparently normal by the middle of August.

On September 6, after an intraspinal injection of rabbit serum, the quantity of albumin and globulin increased, and the percentage of cells changed from 70 to 80 per cent. mononuclears and to 70 per cent. polymorphonuclears. On September 14, following the reaction to the rabbit serum, the quantity of sugar became suddenly greatly diminished, but subsequently increased to normal and remained so until the last fluid withdrawn on October 8, when it again fell off slightly.

From time to time a web appeared in the fluid. At times it was fine, resembling the web in the fluid from a case of tuberculous meningitis. But at times it was dense and gelatinous, with many branches extending from a central filament to the sides of the tubes.

In all, 133 lumbar punctures were performed. Except at the last two punctures, when a higher level was used, the punctures were made in three interspaces—between the fourth and fifth, third and fourth and second and third lumbar vertebrae, and the back remained in excellent condition. Except for some staining of the skin from the iodin, there was little indication that it had been submitted to such a long-continued ordeal.

The cultures grew readily on ordinary agar either with or without the addition of blood. The *p*H of the mediums did not seem to affect the growth. Mediums of various pH values were tried, ranging from 5.8 to 8.6, with no apparent effect on the growth. We used 2 per cent. glucose veal agar, *p*H 7.6. The cultures grew equally well at room and at incubator temperatures. When the colonies first appeared they were pin-point in size and increased rapidly to perhaps a centimeter in diameter. The discrete colonies were uniformly rounded, with a sharply defined margin and a high center. They appeared moist and glistening, and did not adhere tightly to the medium. In consistency they were soft and not stringy. On slants, they coalesced, forming a thick moist, creamy growth. In color, they were a very pale creamy yellow at first, becoming darker yellow as they grew older. The tops of the older colonies were darker than the rest. They grew less well in beef extract broth, *p*H 7.5, than on solid mediums. They clouded the broth slightly and formed a heavy, creamy sediment in the bottom of the tube. There was no pellicle formation. The cultures did not grow anaerobically. No mycelial formation was noted. The action on sugars was studied by surface and stab cultures in veal agar *p*H 7.4 containing 1 per cent. of the various sugars and 1 cc. Andrade. There was no formation of either gas or acid at the end of more than thirty days in the following sugars: lactose, levulose, mannite, glucose, saccharose, inulin, dextrose, starch and glycerin.

The smears of the cultures were gram-positive in the earlier generations, but smears made from cultures several months old showed many gram-negative forms. In the earlier cultures, the organisms had a deeply staining center, with a narrow ring of less deeply staining cytoplasm. While they varied greatly in size, no large forms, such as were found in the smears of the spinal fluids, were ever seen. In the older cultures, the central material was broken up into granules. Many budding forms were seen in the cultures. There was no evidence of endospores.

The absence of endospores, the reproduction by budding, the absence of mycelium and the failure to form gas in sugars identify the yeast as a torula.

Park and Williams give the following tentative classification of the yeast and so-called intermediate groups.

Somewhat rough studies were made in an effort to find some substance that might have an inhibiting effect on the growth of the torula, with the following results: sodium iodid, 1:500, no inhibition; sodium salicylate, 1:500, no inhibition; quinin, saturated solution, no inhibition; a colloidal silver preparation 1:600, 1:1,000, 1:6000, slight inhibition; magnesium sulphate, 1:500, no inhibition; rochelle salts (tartrates) 1:500, no inhibition; tricresol, 1:1,000, some inhibition; acriflavin, 1:1,000, 1:5,000, complete inhibition, 1:10,000, well marked inhibition. Acriflavin was the only substance of the series which had any definite effect on the growth of the torula, but it was not used in treatment.

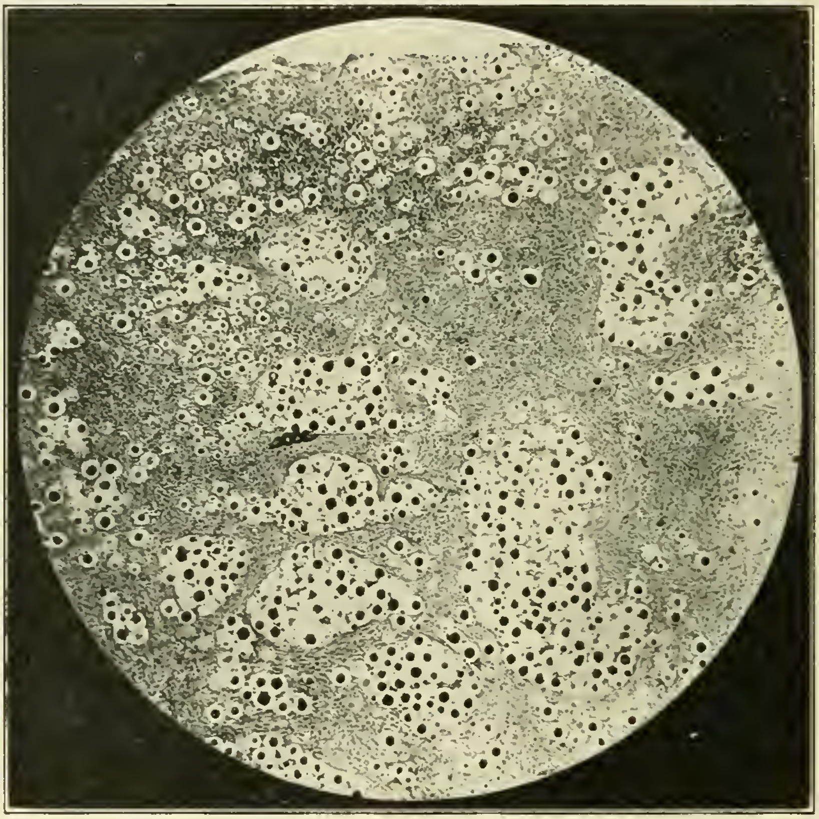

Fig. 5.—Low power magnification of section of subcutaneous mass, guinea pig 644, showing *Torula* arranged in groups. Eosin-methylene-blue stain.

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ANIMAL INOCULATION

On June 8, 1923, eight young animals, four rabbits (about 1,500 gm.) sion of a forty-eight hour culture of the torula as follows: Rabbit 86, one-quarter cc. intracranially; Rabbit 36 one-quarter cc. intracranially; Rabbit 621, five cc. intraperitoneally; Rabbit 829, three cc. intraperitoneally; Guinea-pig 657, one-quarter cc. intracranially; Guinea-pig 655, one-quarter cc. intracranially; Guinea-pig 647, three cc. intraperitoneally; Guinea-pig 653, two cc. intraperitoneally.

Rabbit 86 died July 17, 1923, and no torula were found by culture in any of the organs.

Guinea-pig 657 died June 17, and necropsy was not performed. Guinea-pig 653 died June 22, and showed no torula in the cultures.

Rabbit 829 seemed sick about August 27. This animal had been inoculated intraperitoneally about eleven weeks previously. There was a skin lesion on the back over the lower part of the spine, with superficial ulceration and crust formation. He had general muscular tremors, was ataxic in his movements and seemed to be blind. He was chloroformed and necropsy performed on August 30 by Mary Nevin. Just before death a lumbar puncture was performed, and about 1.5 cc. of spinal fluid withdrawn, which was hazy, showed a slight increase in mononuclear cells and in albumin and globulin and a normal sugar content. The smear showed a few torula and other organisms. The torula was finally recovered in pure culture from the fluid and also from the skin lesion, brain, liver, spleen, lungs and kidney. The organs in the gross showed no definite lesion. The sections showed no large forms of torula, but a study of the sections showed small forms similar to those in the culture scattered through the organs, especially in the periphery of the organs and in the lymph spaces of the brain.

TABLE 2.

PARK AND WILLIAMS' CLASSIFICATON OF YEASTS

	Genus	Endospores	Budding	Mycelium	Gas Formation
Blastomycetes..	Saccharomycetes ...	+	+	−	±
Fungi Imperfecti ...	Torula	−	+	−	−
	Monilia	−	+	±	+
	Oidia	−	+	+	−
	Coccidioides	+	−	+	−

Rabbit 36 died on October 16. It had been inoculated intracranially more than four months previously. It had not appeared sick at any time. It had apparently grown normally since the inoculation and was well nourished. On necropsy, the brain apparently was normal. The liver was pale, mottled and brittle. The spleen was enlarged and very dark. The kidneys were normal. The lungs showed edema, with some hemorrhagic spots and congestion. The torula was obtained in pure culture from the brain, liver, spleen, lungs and heart. The plates were inoculated by smearing sections of the organs over them. The colonies were numerous, and there was no contamination. As in Rabbit 829, no large forms of torula were found in the sections of the brain, but small forms were found as in Rabbit 829. The other organs were not sectioned. It will be observed that in Rabbit 829, inoculated intraperitoneally, the torula had invaded not only the abdominal viscera and lungs, but also the spinal fluid and brain. In Rabbit 36, inoculated intracranially, the torula had invaded not only the brain, but also the abdominal and thoracic viscera. It is also interesting to note that Rabbit 36 lived more than four months and, in spite of the widespread distribution of the torula, grew normally in size, was exceptionally well nourished and showed no evidence of infection up to the time of his death. The fourth rabbit apparently escaped or overcame the infection. He died, Jan. 18, 1924, over seven months after inoculation, and no torula were recovered by culture from any of the organs.

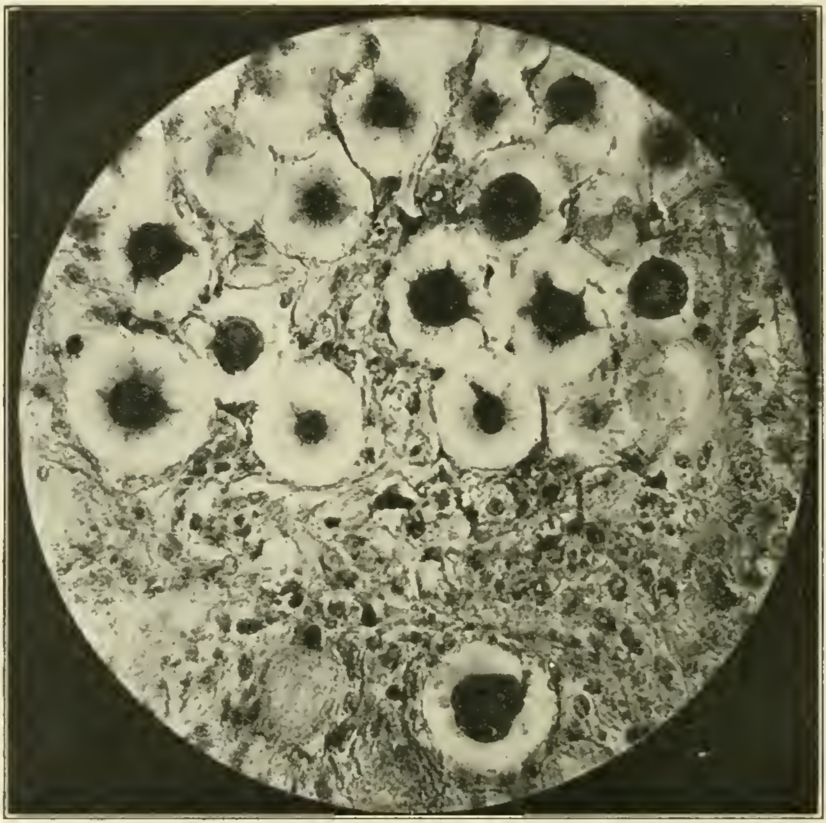

Fig. 6.—High power magnification of section in Figure 5. There are numerous *Torula*—a few budding forms. (Reprinted by courtesy of Archives of Neurology and Psychiatry.)

The two guinea-pigs that survived grew rapidly and seemed perfectly well. They were kept under observation, however, and on November 3, more than five and one-half months after inoculation, it was noticed that Guinea-pig 655, inoculated intracranially, showed spasmodic contraction of groups of muscles, and held the head turned markedly to one side. He was weak in in his movements and was unable to stand normally. He was panting for breath. He was chloroformed on that day and necropsy was performed. The brain was edematous and soft, and the fluid oozed out of it. The right lung was small and shrunken; the left was swollen. The spleen was small and mottled. The stomach was empty and distended with gas. The other organs seemed normal. The torula was obtained in pure culture from the brain, lungs, liver, spleen, kidney and heart. The plates were inoculated by smearing with pieces of the organs. The colonies were numerous, and the culture showed practically no contamination.

In the sections from Guinea-pig 655, many large forms of the yeast were found, especially in the lymph spaces of the brain and in the meninges, and also in the lung (Figs. 7 and 8). It will be noted that budding forms are also seen.

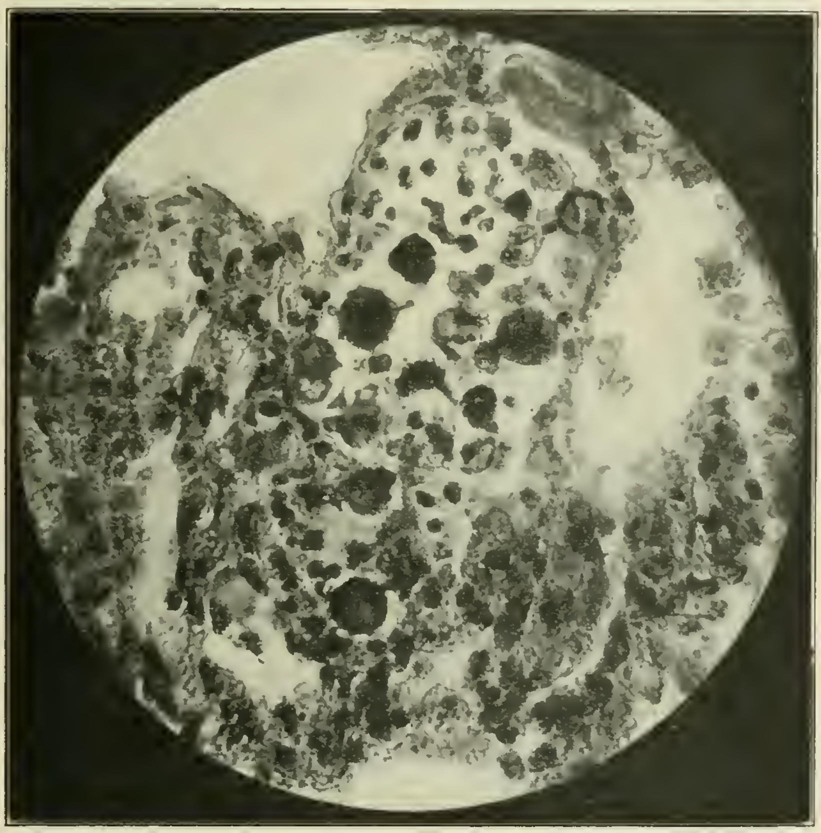

Fig. 7.—High power magnification of section of lung of Guinea-Pig 655. There are numerous *Torula;* a few budding forms.
(Reprinted by courtesy of Archives of Neurology and Psychiatry.)

Guinea-pig 644 seemed normal until late in December, when a hard mass was observed in the left abdominal wall. Soon after that, small ulcerative lesions were observed on the right lower eyelid and on the right cheek. He was chloroformed and necropsy performed on Jan. 3, 1924, nearly seven months after the intraperitoneal inoculation. The brain and other organs seemed normal, and no torula could be cultivated from them. The torula was cultivated from the mass in the abdominal wall, and from the two skin lesions. The plates were inoculated as in the previous cases. The colonies were numerous and the culture practically pure. All these cultures were tested with the sugars (mannite, glucose, glycerin, starch, saccharose, dextrin, inulin, lactose and levulose) and gave no fermentation with any of them. The mass in the abdominal wall was located in the subcutaneous tissue between the skin and the abdominal muscles. It was about 2 inches in length and 1 inch in diameter. It was irregularly spindle-shaped and about the consistency of paraffin. In the sections of this mass many large forms of torula were present. The arrangement in masses is shown in Figures 5 and 6. It will be noted that there is practically no cellular reaction accompanying the invasion with the torula. The torula was much better shown by the eosin-methylene blue stain than by the hematoxylin-eosin.

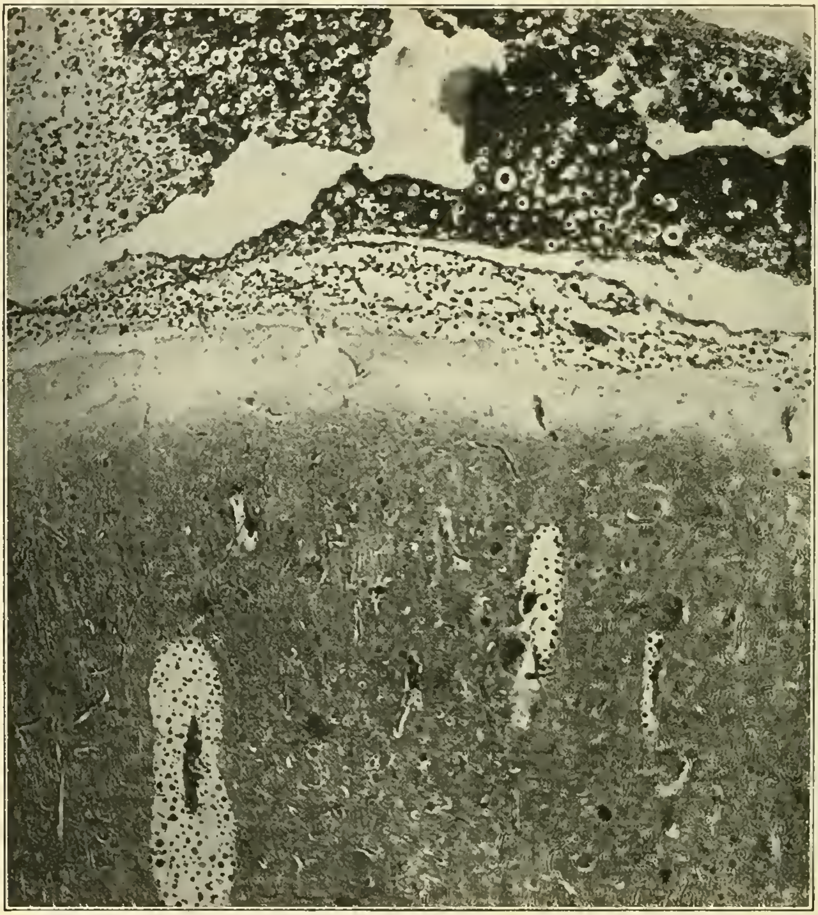

Fig. 8.—Low power magnification of section of brain of Guinea-Pig 655, showing *Torula* in lymph spaces around blood vessels and in meninges. Eosin-methylene blue stain. (Reprinted by courtesy of Archives of Neurology and Psychiatry.)

CONCLUSIONS

In comparing our case with those reported by others, it may be pointed out that we were fortunate in having the patient under our observation during the entire course of the illness. The diagnosis was made within the first two weeks of the acute illness and within five days of the onset of symptoms involving the central nervous system. It is possible that the real onset of the infection was in February, with a period of quiescence lasting until May. He lost 15 pounds in weight during this time, although otherwise apparently well.

Various methods of treatment were tried, apparently with no effect. Arsphenamin had been used by Stoddard and Cutler without success in one of their cases, and was not tried in our case. Decompression was considered, but the repeated lumbar punctures seemed to afford sufficient relief of pressure.

In spite of the fact that the boy was acutely ill for five months, with a constant fever, it was possible to keep him exceptionally well nourished until the very end, and there were very few focal symptoms, although the disease must have been constantly progressing. These same points were observed in the animals inoculated as has already been mentioned.

From a study of the patient and the animals inoculated, infection with this torula seems to be unusually slow and insidious in its course, and inevitable in its outcome.

For bibliography see the original article.

A CASE OF SPOROTRICHOSIS MENINGITIS

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George H. Hyslop, Josephine B. Neal, Walter M. Kraus and Oliver Hillman

Introduction

We wish to report this case of chronic meningitis because the spores constantly found in the spinal fluid of the patient were morphologically similar to the sporotrichia of Schenck and de Beurmann. We feel satisfied that these organisms were the specific cause of the infection, and that our diagnosis was warranted, although we failed to obtain positive cultures and our animal inoculation experiments were inconclusive.

Sporotrichosis in man was first described by Schenck in 1898. Following him, de Beurmann and Gougerot reported several cases in 1900. Since then various reports have appeared, so that the disease has become well established and easily recognized clinically.

The infection is due to the sporotrichia, an organism allied to the hyphomyces. It is pathogenic for horses, dogs, rats, rabbits and guinea pigs. In man, it commonly produces skin lesions which are gummatous in character and may be mistaken for syphilitic or tuberculous lesions. The swellings are usually painless, slow in growth, chronic in nature, and they may either show a central necrosis, eventually breaking down with sloughing of the overlying skin, or they may resemble indolent ulcers.

The infection may become systemic, and it has been reported to have involved the lungs, bones, periarticular tissues, kidneys, and the eyes, causing miliary conjunctivitis and occasionally panophthalmitis.

Involvement of the central nervous system in man has never been reported. Dominguez, however, cites a case of an extradural abscess, complicating a sporotrichosis infection of the middle ear.

The organism is difficult to find in the tissues, but when present is pleomorphic. In smears from the tissues, the sporotrichia may be seen as round or oval bodies with dimensions from 1 to 2 micra by 3 to 5 micra, and appear occasionally in bacillus-like forms.

It grows at room or incubator temperature, and best in sugar media (dextrose, levulose and maltose). Sabouraud's medium is commonly used. It is also reported to grow in 1 per cent. acid broth. First cultures may be slow in appearance and may not be visible for as long as two weeks. They are white or dirty gray on solid media, but if the blood media are used, the cultures may be brown or black pigmented. Microscopically, the growth usually shows branching filaments, with spores in the filaments or in the interspaces. Occasionally the filaments radiate as they do with actinomyces. Subcultures grow more luxuriantly.

A definite diagnosis is made by positive cultures. Complement fixation, agglutination, and skin reaction tests have diagnostic value.

Iodides are specific for the disease.

Case Report

Past History. The patient was a girl aged fifteen years, of American stock and a resident of Bayonne, New Jersey. She had had mumps, measles and scarlet fever, and in 1923 she suffered from what was called a mild attack of malaria. Menstruation began at the age of thirteen years, and was normal in character.

During the fall of 1924 she was in school and in perfect health. Just before Christmas she fell down a flight of stairs, without injury other than bumping the back of her head; she was not even stunned.

Present Illness. On December 30, 1924, she became ill with nausea, vomiting, headache, generalized abdominal pain, and a fever which was not higher than 103°F. In a few days, photophobia, weakness of the right side of the face and intense drowsiness developed. The fever disappeared after three or four days, but the nausea and vomiting coninued, and her headaches were quite severe.

About January 15, 1925, diplopia, strabismus, and lancinating pains in the shoulders appeared. During the next month, symptoms fluctuated from day to day, with a trend toward improvement. The girl could not walk well, because of a tendency to stagger to the left, and any sudden change of position was apt to cause projectile vomiting. She was emotionally unstable, and had frequent nightmares.

She was examined in consultation on February 19. She seemed perfectly normal mentally, and coöperated well. The pulse was 100, there was no fever, and body nutrition was unimpaired. There were no signs of visceral disease.

She preferred to lie in bed, but could sit up comfortably if she moved carefully. With one eye covered she could walk perfectly well.

Cranial Nerves. There was obliteration of both optic disks and moderate engorgement of the retinal vessels; both pupils were large and irregular, but reacted to light and accommodation normally. The left external rectus muscle was completely paralyzed, and the right was partially so. There was a mild lower neurone type weakness of the right facial muscles. Otherwise the cranial nerves were normal.

Motor System. All tests were performed well, except for a cerebellar incoördination in the left arm and leg.

Sensory System. No abnormalities were found.

Reflexes. Brudzinski and Kernig signs were positive. The arm and abdominal reflexes were normal. The knee and ankle jerks were abolished, and the plantar responses were sluggish and equivocal in type.

From February 19 to March 5 there was a steady improvement. There was almost no headache, no drowsiness, a decrease in vomiting, and she was able to go about the house. On March 5, a day or two after some difficulty in naming persons and objects had been observed, she vomited several times. While talking in the afternoon with some friends, she suddenly cried out, saying "I can't, I can't," and in a moment became flushed, clenched her arms and was unable to speak. In a few minutes she recovered sufficiently to get to her room. There was considerable vomiting for several hours, and it was 10:30 p.m. before she could express herself in speech. She seemed frightened, misnamed her mother, and talked incoherently of being cut and hurt. By the next morning she was normal mentally and showed no aphasia, but there was considerable headache, and projectile vomiting occurred if she moved about in bed.

Examination on March 6 showed that there had been an improvement in the external rectus palsies with no change in the other abnormalities found at the time of the first examination. However, there were three new signs—a left partial ptosis, tinnitus in the left ear, and exudate in the left retina. The retinal vessels were moderately dilated, the disk was not visible, and radiating from the edges of the disk were large splashes of pure white exudate, extending outward into the retina a distance of perhaps two disk diameters. There was enlargement of the blind spot in the left eye, but no impairment of the visual fields. The right fundus was normal. There were no evidences of an aphasia or other residuals of the attack.

On March 10 another attack with confusion and a loss of speech, occurred. The patient had been drowsy the day or two preceding. She was then admitted to the Neurological Institute.

Lumbar puncture was done, and about 40 cc. of fluid was drawn. It was clear and under intense pressure. Physical examination revealed increase in the right facial weakness, and much impairment of vision in the left eye, so that fingers could be counted only when held at a 6-inch distance. The neck was quite stiff. Otherwise there were no changes since the examination of March 6.

Blood count showed 21,000 white cells, of which 82 per cent. were polymorphonuclear leukocytes. The urine was normal.

The spinal fluid contained two cells and 160 spores per cubic millimeter. Globulin and albumin were 2 +; and sugar, 68 mg. per 100 cc. Serological tests were negative. A smear of the centrifuged fluid, stained with methylene blue, showed abundant round and oval bodies which took the stain well and in many instances contained what seemed to be polar bodies. There were a few phagocytes containing small inclusions, and an occasional lymphocyte (see Figs. 1 and 2). Cultures of this specimen of fluid were negative.

The bodies seen in the smear were identified by Dr. Anna Williams, of the Department of Health, as sporotrichia spores.

Within a few days the left eye became blind. The patient had severe headache much of the time, and cerebral vomiting was frequent. Lumbar punctures were done at least once a day to relieve the meningeal irritation symptoms, and from 40 to 60 cc. of fluid was drawn each time with, as a rule, immediate improvement, lessening of the headache, decrease of the drowsiness, and cessation of vomiting. There was a slight fever, the rectal temperature varying between 99° and 101°F.

During the next three weeks no new clinical phenomena appeared, but there seemed to be periods of subsidence of all symptoms, alternating with periods of exacerbation lasting two or three days. With the increase of meningitic signs the spinal-fluid pressure would be greater, there might be a slight fever, and the blood count would show a leukocytosis of from 14,000 to 20,000 cells, the polymorphonuclears ranging from 82 to 90 per cent.

On April 10, routine examination of the fundi showed an unusual picture in the left retina. Previously, the right retina exhibited a mild papilledema and slightly congested vessels, and in the left retina were the same splashes of peripapillary exudate that were first observed on March 6, dilated veins with occasional hemorrhages near the disk, and a nerve-head elevated about 4 diopters. Now to the temporal side of the left optic disk appeared a glistening pearly-white elevation, with borders serrated as are the edges of a cumulus cloud. This mass was 6 diopters in elevation, and across its surface ran small veins and arteries which dipped down at the edges and passed along with the retinal vessels. The retinal vessels were very tortuous, and there were large numbers of small hemorrhages scattered over the entire retina. This pearly-white mass seemed to be utterly unlike what one observes in a detached retina, and was regarded as of inflammatory origin.

With the exception of a period of two and a half weeks in April, the course of the disease seemed to be uninfluenced by any treatment. It was characterized by periods of relapse every seven to ten days, and in the last month by a gradual but steady increase in the signs of a posterior fossa meningitis. As a rule there was no fever, but during the relapses the temperature at times reached 101°F. Death came on June 19, five months and three weeks after the onset of the illness.

From the clinical standpoint there were several important features which must be considered.

From March 5 until the termination of the illness on June 19, a period of three and a half months, there were few new signs indicating extension of the disease process.

Roentgen ray of all the bones of the body showed no evidence of any infection with the sporotrichia. Roentgen ray of the gastro-intestinal tract was negative. The lungs showed a moderate degree of bronchial fibrosis. Cultures of saliva, sputum, naso-pharynx and tonsil smears, urine, blood and feces were negative for sporotrichia.

Roentgen ray of the skull revealed a thinning of the vault associated with a long-continued increase in intracranial pressure.

I. Spinal Fluid. The pressure of the spinal fluid was constantly and markedly increased. On one occasion manometric reading showed a pressure of over 600 mm. water. Lumbar punctures were done daily for therapeutic purposes.

The table indicates the variations in the fluid found on routine examinations. As a rule from 40 to 60 cc. were drawn at each tap. There were only 20 to 30 lymphocytes to the cubic millimeter, although the fluid was usually blood-tinged and contained red cells in amounts from a few hundred to 2000 or 3000 per c.mm. The spinal fluid was as a rule more bloody during the relapses, which would last two or three days.

Following two intraspinal injections of sodium iodid, there was an exacerbation of meningitic signs, and, for a few days, the spinal fluid contained about 5000 white cells per cubic millimeter, of which nearly 100 per cent. were polymorphonuclear cells.

Wassermann and colloidal-gold tests were negative. No tubercle bacilli were found at any time, and guinea-pigs inoculated showed no evidence of tuberculosis.

On standing at room temperature or in the incubator, the fluid would at times become opalescent.This phenomenon was unfamiliar to us and we were unable to determine its cause or chemical nature.

Hanging drop specimens from a large number of samples of spinal fluid were examined. In every specimen were visible small oval bodies which were approximately 2 or 3 micra wide and 3 to 5 micra long. These were refractile, and many of them seemed to contain at the smaller end what might be taken for a polar body. They exhibited a peculiar sort of motion. It was a combination of Brownian movement, with a revolving about the long axis of the body. This motility would decrease during examination and was

TABLE—SPINAL FLUID ANALYSIS

Color and date of appearance.	Lymphocytes per cubic millimeter.	Erythrocytes.	Globulin.	Sugar (per cent.)	Hanging drop.	Stained smears.	Cultures
March 10. Clear, colorless	2	0	2+	0.068	160 spores	Spores, no other organisms	Negative to 21 days
March 13. Clear, few flocculi	3	0	2+		40 spores	Negative	Negative to 21 days
March 14. Clear, colorless	20	0	2+	0.068	20 spores	Spores	Negative to 21 days
March 15. Hazy, yellowish	8	Many	2+		72 spores	Spores	Negative to 21 days
March 18. Hazy, yellowish	9	A few	2+	0.051	Few spores	Spores	Negative to 21 days
March 19. Clear, colorless	2	0	3+	0.088	Few spores	Spores	Negative to 21 days
March 21. Cloudy, fibrinous sediment.	4600*	A few	4+	0.048	Few rods	Spores	Negative to 21 days
March 23. Yellowish, bloody	Many	Many	4+		Spores and filaments	Spores and filaments	Mold contamination
March 26. Cloudy, blood tinged	25	Many	2+	0.040	Spores and rods	Spores and filaments	Negative to 21 days
March 30. Slightly hazy	....	....	....	....	Spores and rods	LargeGram-plus rods and spores	Negative to 21 days
April 1. Clear, colorless	0	Occasional	1+		Spores	Spores	Negative to 21 days
April 9. Clear, yellowish	20	Few	3+		Spores	Spores	Mold and Staphylococcus contamination
April 14. Hazy, yellowish	30	Many	2+	0.042	Spores	Spores	Negative to 21 days

* Seventy-seven per cent. polymorphonuclears, 15 per cent. lymphocytes, 8 per cent. arachnoid mononuclears.

NOTE.—The spores were found easily in the hanging drop specimens, but with difficulty in smears of sediment or of supernatant portion of centrifuged spinal fluid. We cannot account for this. We neglected to vary the specific gravity of specimens of spinal fluid before centrifuging them.

not found when the spinal fluid had been standing for some time. The motility of these bodies was not due to convection currents, because the bodies would move about the field, whereas red or white cells would remain

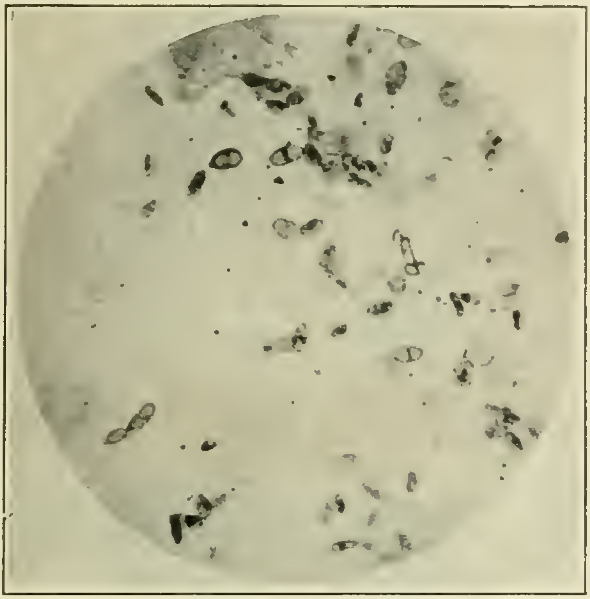

Fig. 1.—Spore forms in spinal fluid smear. (Methylene blue stain.)
(Reprinted by courtesy of American Journal of the Medical Sciences.)

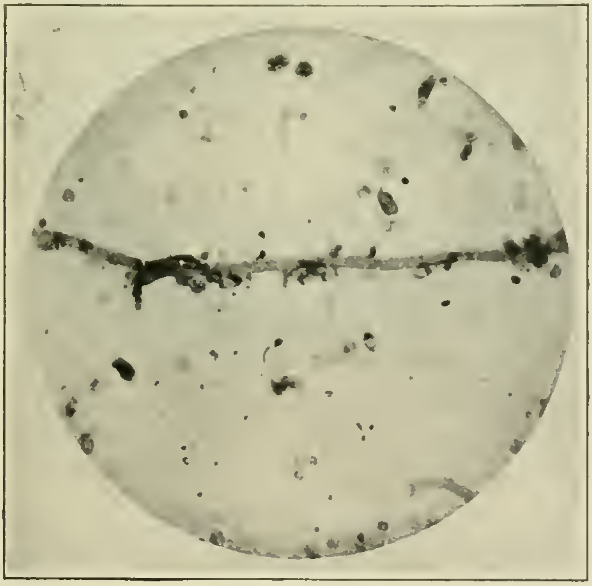

Fig. 2.—Spinal-fluid smear (oil immersion) showing mycelial filament and spores.
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stationary. Occasionally one could see a macrophage, with an ameboid movement, and containing several dark round inclusions. The motile bodies were in a few instances paired end-to-end and once a chain of three was found.

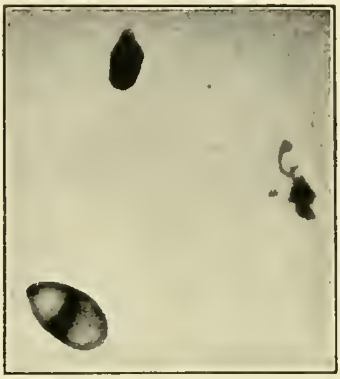

Fig. 2*a*.—Spinal-fluid smear. Oil immersion × 10. Shows dividing form.
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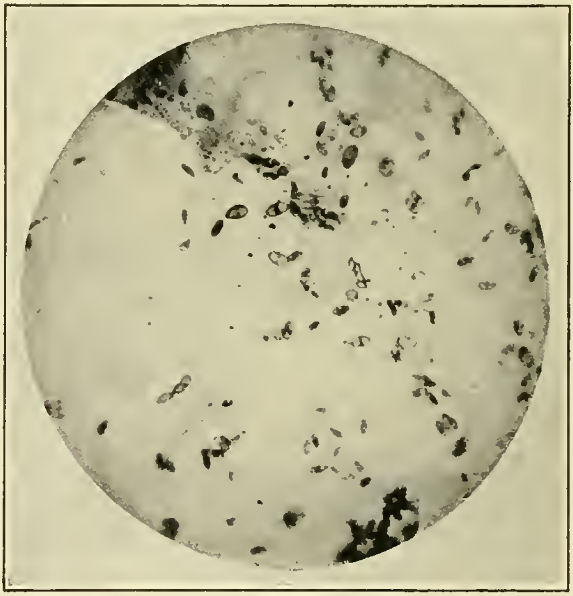

Fig. 2*b*.—Spinal-fluid smear. Shows spores, varying sizes and dividing forms.
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Perhaps half a dozen times rod-like forms with Brownian movement were seen.

Stained smears of the centrifuged sediment of spinal fluid were made. About twenty slides from as many different specimens of spinal fluid were examined (see Figs. 1 and 2). Careful search of each smear revealed in every instance the presence of spore-like bodies. Mycelial forms occurred in two or three of the smears. These bodies stained readily and were Gram amphophilic. Dr. Anna Williams of the Department of Health regarded these bodies as belonging to the pathogenic molds, and stated that they were probably spores of the sporotrichia.

It was observed that these bodies were less numerous in the centrifuged sediment than they were in the hanging drop specimens made from fresh spinal fluid.

Cultures were made from many samples of the spinal fluid, in the laboratory of the Neurological Institute and also in the laboratory of Dr. Neal. Aërobic and anaërobic cultures were made and a large variety of sugar, blood and broth media were used in attempts to grow cultures at both incubator and room temperatures. We failed to secure any positive cultures. Both mold and bacteria-contaminating organisms were occasionally found. It is impossible to explain why these numerous attempts at cultures failed, especially when the sporelike bodies were constantly found in smears made of the centrifuged sediment of spinal fluid.

Dr. Elser (personal communication to one of us), has observed one case of skin sporotrichosis in which positive cultures could not be obtained, and he considered it possible that iodid medication might have affected the organism so that it would not grow in culture.

II. Tetanoid Attacks. From March 16 to May 25 there occurred occasional mild attacks of tingling in the extremities, associated with spasm of the muscles of the hands, which assumed the accoucheur position. From May 25 until death, such attacks occurred almost daily and, when more severe, there was extension of the legs and trismus. Myoclonic movements of the abdominal muscles accompanied these tetanoid attacks during the last few days of life.

III. Temporal Lobe Phenomena. The first occasion on which it seemed that the temporal lobe was involved was during the attack of confusion and aphasia on March 5. This attack and the one on March 10 were the most definite instances. However, in the last month of her illness, the patient frequently would become suddenly confused and lethargic, and for perhaps an hour would exhibit a mild paraphasia.

During two or three of the relapses in which the patient became somewhat disorientated in time and place and became quite forgetful, microptic visual hallucinations were present. These hallucinations were characteristic and were linked in this patient with disturbed function on the left cerebral hemisphere. The reason for considering the hallucinations as due to a pathological process in the left hemisphere is that the little people appeared consistently in the right half of the visual field. This could be determined not only from questioning the patient, but by observing the patient's behavior when the hallucinations were present.

IV. Treatment. When it seemed that we were dealing with a sporotrichosis infection we began intensive iodid therapy. From about March 15 until May 20, the patient received from 60 to 150 gr. of sodium iodid a day. During March it was given intravenously as well as by mouth; twice it was administered intraspinally. The intraspinal injections caused a great deal of pain, and also produced a rather severe aseptic meningitis. A few injections of salvarsan were given over a period of three weeks in May and early June. Quinin was given in March and discontinued after two or three days because the patient did not tolerate it. It was tried again after the iodids were discontinued and had to be given up for the same reason.

In addition to the above drugs, symptomatic remedies were used. Of these, retention enemas of 25 per cent. salt solution seemed definitely helpful in relieving the vomiting, which was much more frequent during the relapses, and at times when the spinal-fluid pressure was greatest.

Since the sporotrichosis infection produces granulomatous lesions, since we were dealing with a very serious situation, and since it has been observed that intravenous administration of a solution of active deposit or radium emanation has at times had therapeutic effect in Hodgkin's Disease, we felt justified in trying an experiment.

On April 2 the patient was given intravenously 70 millicuries of active deposit solution of radium emanation. At the time this was given the patient had been moderately deaf for about two weeks, so that whispers could not be heard and the patient was unable to hear the children singing in a nearby school or detect the sounds of people passing through the corridor by her room. She had a bilateral facial weakness, there was right internal strabismus, and the signs of meningeal irritation had been troublesome for several days.

The injection was given at 1:30 p.m. At 3 p.m. the patient vomited twice. At 4 p.m. tenderness and swelling of both parotid glands appeared. By 6 p.m. the swelling had extended to the submaxillary glands and also involved the lateral and posterior cervical lymph nodes. The patient was also quite drowsy and complained bitterly of a stiff neck. During the night she observed to the nurse that she was again able to hear sounds which she had been unable to hear for a week. The next morning the meningeal signs were markedly reduced and less than they had been for some days. During the next few days the salivary glands and lymph node swelling disappeared, the eye movements were better, the facial weakness improved, and the spinal fluid gradually became free of red blood cells and the sporelike bodies became scanty in numbers. From about April 10 to 30, the patient's general condition steadily improved and the cranial nerve signs also lessened. The patient became able to walk about the room, stay up most of the day, and even took a few rides about the town in an automobile.

During May when the patient had become worse, another dose of active deposit was given. The same swelling of the salivary glands ensued, and there was also a mild systemic reaction with a temporary exacerbation of the meningeal signs. However, this injection was not followed by the clinical improvement which occurred after the first injection.

It is interesting to speculate upon the phenomena following the first injection. In particular, the swelling of the salivary glands was noteworthy. This complication has not been observed in a series of several hundred intravenous injections of radium deposit at the Memorial Hospital in New York. It is conceivable that the glands became swollen because they were the avenue of entrance of the sporotrichia infection, and, therefore, were already predisposed to the reaction which would be a consequence of the radium attacking infected tissues.

It is also conceivable that the radium, in producing a systemic reaction, upset the vegetative function and caused a temporary disturbance in the gonads. The injured gonads may have then caused a selective sympathetic involvement of the salivary glands, exhibiting in the reverse fashion the relation ship between the salivary glands and the gonads, well known in mumps.

Necropsy. A complete necropsy was done six hours after death. Examination of the viscera showed no abnormality other than might be found due to agonal causes, and nothing which would throw light on the lesions in the central nervous system. The skin showed no lesions of any description, and the mucus membranes were normal with the exception of a mucopurulent conjunctivitis in both eyes. This appeared a few days before death.

The gross pathology was limited to the intracranial cavity. Protocol of the examination of the head and intracranial cavity follows:

Head. Symmetrical and not enlarged. Scalp and pericranium negative. Dura slightly adherent to calvarium; latter thin over vertex. Sutures quite visible. Superior longitudinal sinus empty. Dura everywhere appears normal.

Brain. No bulging. Cerebral hemispheres apparently symmetrical. Veins moderately prominent, but there is no marked convolutional capillary congestion. Convolutions over vertex somewhat flattened and sulci compressed. Pacchionian bodies small. Piaärachnoid over parieto-temporal regions semi-opaque and slightly thickened. Cerebellar convolutions fairly well obliterated, particularly on the left side. There is considerable cloudy, bloody fluid in the subtentorial space. Base of brain shows widespread subarachnoid accumulation of cloudy fluid—well marked between the cerebellar hemispheres, where a gelatinous mass 5 by 1½ by 1 cm., is seen The piaärachnoid appears thickened over the inferior surface of the cerebellum and in the interpeduncular space. An occasional small area of hemorrhage is seen in the leptomeninges over the base. No granulomatous tissue, abscess formation, or softening of the cortex observed. There is a gelatinous exudate about the left optic nerve, and in the left orbit, where there is also considerable edema of the orbital fat. Retinæ were not removed.

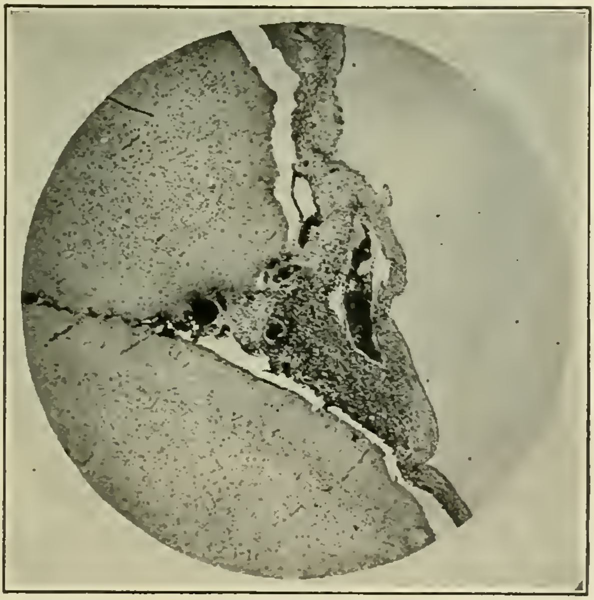

Fig. 3.—Showing leptomeningitis, temporo-parietal cortex. (Hematoxylin-eosin stain).

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There is slight internal hydrocephalus as determined by puncture of the ventricles, 10 cc. being withdrawn from the left and 5 cc. from the right. Fluid slightly hazy.

The ethmoid, sphenoid, frontal and mastoid sinuses and the middle ears were opened and were all found to be perfectly normal and free of any fluid, mucus, or inflammatory thickening of the membranes.

On section, after hardening in 10 per cent. formalin, the brain showed softening of both basal ganglia, probably postmortem. There was a very marked thickening of the piaärachnoid and of the ependymal lining of the ventricles. The spinal cord was not removed.

Anatomic Diagnosis. Chronic diffuse leptomeningitis, with gelatinous exudate beneath piaärachnoid. Exudative periophthalmitis and perineuritis (left).

Histologic Examination. [Stains: Hematoxylin and eosin, Mallory, Nissl, van Gieson and Loyez (myelin)].

Temporo-parietal Area. There is a marked thickening of the pia-arachnoid with increase of connective tissue and great infiltration of the pia with numerous lymphocytic and plasma cells (Fig. 3). In the cortex there is well-marked perivascular edema with occasional scattered round cells in the Virchow-Robin spaces (Fig. 4). There is edema of moderate degree in the outer layers of the cortex, but no hemorrhages. No degenerative changes of ganglion cells beyond scattered very slight swelling of isolated cells in the outer layers of the cortex. No glia proliferation. In the subcortical region there are numerous intraglial foci filled with metachromatic lipoids, but there is no degeneration of the myelin sheaths.

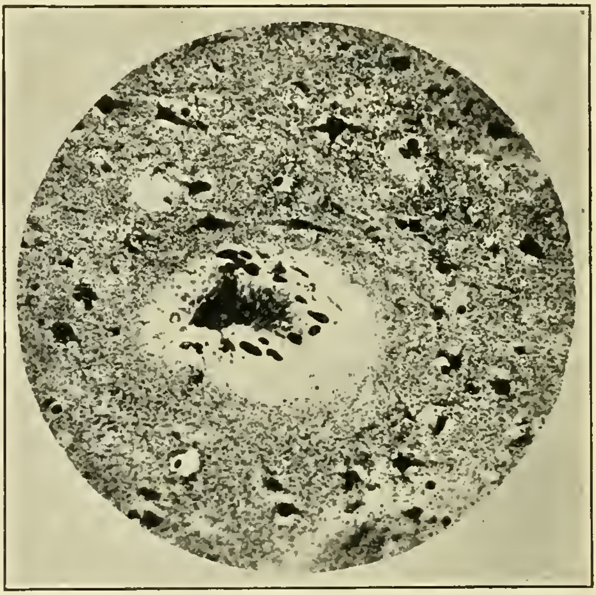

Fig. 4.—Perivascular infiltration of temporo-parietal cortex. (Rio Hortega stain.)
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Cerebellum. The picture of the meninges covering the cerebellum is similar to that described (cerebral). The perivascular edema within the cerebellum is of minor degree with absence of cellular infiltration. Foci of the mycelium forms with spore inclusions were found in the meninges covering the cerebellum.

Medulla. The leptomeningitis is also found surrounding the medulla oblongata. The Loyez (myelin) stain shows swelling of the myelin sheaths throughout the whole section, most pronounced at the periphery where transitions to partial myelin sheath degeneration and complete loss of myelin sheaths are noticed.

HISTOLOGIC DIAGNOSIS. Severe chronic leptomeningitis and low-grade cerebral edema.

ANIMAL INOCULATIONS. The animal inoculations were carried out in the laboratory of Dr. Josephine B. Neal. The results follow:

A. FROM SPINAL FLUID OF PATIENT. *Mice.* March 19. Two mice injected intraperitoneally; mice living three months later.

March 22. Three mice injected intraperitoneally. One died of pneumonia, one died of brain hemorrhage and one died of causes unknown.

March 29. Three mice injected intraperitoneally. One mouse living, two mice on necropsy two weeks later had tapeworm and also showed enlarged and reddish lymph nodes, adherent viscera and deposits of gelatinous substance in the peritoneum. Cultures negative.

April 14. Nine mice injected intraperitoneally; living four months later.

Guinea Pigs. March 19. Two guinea-pigs injected intraperitoneally. Both died of pneumonia and cultures showed pneumonococci.

March 20. Two guinea-pigs injected intraperitoneally. One of the guinea-pigs died of tuberculosis.

March 22. Two guinea-pigs injected intraperitoneally. One guinea-pig showed, on necropsy, enlarged inguinal and axillary lymph nodes, and peritoneal and pleural fluid; cultures were negative. The second guinea pig on necropsy, showed mottled lungs, normal kidneys and liver, and gelatinous masses in the peritoneal cavity. Cultures were negative.

June 9. Two guinea pigs injected intracranially. One died in twenty-four hours presumably of shock; the other guinea pig is alive.

Rabbits. June 9. Two rabbits injected intracranially. Both rabbits died of septicemia which was passed to subculture guinea pigs. The cultures showed cocci and bacilli.

B. VENTRICULAR FLUID (POSTMORTEM ON PATIENT). June 19. Three guinea pigs injected intraperitoneally. One guinea pig is alive. Another guinea pig died July 11; the brain was found to be congested. Cultures negative. The third guinea pig died July 8 and showed congested brain and lungs; cultures were negative.

Subcultures on two guinea pigs from this third animal gave negative results.

C. EMULSION OF BRAIN SWABS OF GELATINOUS EXUDATE (FROM BRAIN OF PATIENT). June 19. Four rabbits injected intracranially. Two died of injection shock; one died of septicemia, which was passed to guinea pigs; one at death showed hemorrhage of the brain, cultures of which were negative.

June 19. Three guinea pigs injected intraperitoneally. One is alive, another guinea pig died in September of pneumonia, and a third died three days after inoculation. Necropsy was not done.

D. RÉSUMÉ OF EXPERIMENTAL WORK. Six rabbits, 14 guinea pigs and 17 mice were inoculated. Of these, 2 mice and 1 guinea pig, which were inoculated intraperitoneally with spinal fluid from the patient, showed a gelatinous exudate in the peritoneal cavity. This exudate was, in appearance, similar to that found under the meninges of the patient.

Inoculations of postmortem material from the patient gave results which were not helpful.

Examination of the gelatinous exudate found in the mice and guinea pigs revealed no organism.

Histologic study of postmortem material of inoculated animals was not helpful and corroborated the clinical diagnosis as to cause of death only.

SUMMARY. Sporotrichosis infection of the nervous system has not been observed previously.

Our patient was a girl, aged fifteen years, who became ill December 30, 1924, and died June 19, 1925. The infection involved the posterior fossa largely, had a febrile onset, and ran a chronic remitting course. There were slight exacerbations about every ten days, accompanied by a slight fever.

Interesting clinical occurrences were tetanoid attacks, microptic hallucinations, and several temporosphenoidal seizures.

The diagnosis was made by the presence of sporotrichia spores and mycelial forms in the spinal fluid.

Iodid therapy was of no avail. Experiments with radium intravenously gave interesting results.

Culture and animal inoculation results were negative.

Microphotographs of the spores seen in the spinal fluid and of the tissue from the patient's brain are exhibited.

Autopsy of the patient showed a chronic diffuse leptomeningitis most marked in the posterior fossa, a moderate edema of the brain and the presence of a peculiar gelatinous exudate here and there beneath the pia. Microscopically, there was shown a chronic productive leptomeningitis but no inflammatory involvement of the brain substance.

For bibliography see the original article.

STREPTOCOCCIC MENINGITIS FOLLOWING SCARLET FEVER—RECOVERY

Published in The Archives of Pediatrics, 1927, Vol. XLIV, pp. 395-398

By Josephine B. Neal and Archbold Jones

The mortality in streptococcic meningitis is so high that it is a matter of interest when a patient suffering from it recovers. This case is one of particular interest because there is reasonable ground for believing that the treatment was specific.

A. P., a girl 7 years of age, was seen by Dr. Emanuel Appelbaum of the Meningitis Division of the New York City Health Department on January 30, 1927. There was a history of a mild attack of scarlet fever beginning on January 20, 1927. The symptoms lasted only two days and the following two days she seemed perfectly well. The meningeal symptoms then developed suddenly with fever and headache. Her condition became decidedly worse on the morning of January 30.

On physical examination many of the typical signs of meningitis were present. There was marked stiffness of the neck and a positive Kernig and Brudzinski sign. The pupils were equal and reacted to light. The fundi of the eyes were normal. The patella reflexes were absent; the biceps and abdominals were diminished. There was a Babinski sign on the left but the confirmatory signs were absent on both sides. There was a left otitis media. She was clear mentally. The heart, lungs and abdomen were normal. The temperature was 103°F.; pulse, 100; respiration, 20. On lumbar puncture, 60 cc. of purulent fluid, under greatly increased pressure, was withdrawn and 20 cc. of anti-meningitis serum was injected, although the history of scarlet fever and an otitis media suggested the strong possibility of a streptococcic meningitis. The examination of the spinal fluid showed a very great increase in cells, practically all polymorphonuclear. There was a moderate increase in protein and an absence of sugar. No definite organisms were demonstrated in the smear but the culture showed a hemolytic streptococcus.

The patient was admitted to Willard Parker Hospital late at night on January 30, presenting much the same clinical picture. On account of the history of scarlet fever and the presence of desquamation, 30 cc. of antiscarlatinal serum No. 14 was given intramuscularly. This is a combined antitoxic and antibacterial serum prepared under the direction of Dr. W. H. Park and Dr. A. W. Williams and described by them as "Antiscarlatinal Serum of Dual Potency." (Proceedings of Soc. of Exp., Biol. and Med., 23; 2839, 1925).

On the morning of January 31 a lumbar puncture was performed at the Hospital and, as it was not known that the previous fluid had been cultured, antimeningitis serum was given again. This spinal fluid showed Gram positive cocci by smear and a hemolytic streptococcus by culture. Later that same day another puncture was done and about 15 cc. of scarlatinal serum No. 14, containing ¾ cc. of one per cent. neutral acriflavine, was injected. At this puncture only 2 or 3 cc. of spinal fluid could be obtained, indicating that there was perhaps some block developing. The serum ran in with some difficulty. The child seemed to be desperately ill. She was semi-delirious and the temperature was between 104°F. and 105°F.

On February 1 and 2 the condition was about the same. The neck was greatly retracted. Only 1 or 2 cc. of blood-tinged fluid could be obtained by lumbar puncture but 15 cc. of the antiscalartinal serum was injected in part by gentle pressure along the tube of the gravity apparatus. This indicated a block rather high up and the use of the dye was discontinued after the first injection, for it was feared it might increase the tendency to the formation of adhesions. These spinal fluids were sterile.

On February 3 a cistern puncture was easily done, 45 cc. of cloudy fluid being removed and 20 cc. of antiscarlatinal serum being injected. This fluid gave a culture of hemolytic streptococcus, although no organisms were demonstrated in the smear. The protein was somewhat less than in the first two fluids and the sugar was normal, by the Fehling's reaction.

On February 4, a cistern puncture was unsuccessful, due, it was felt, to adhesions, as the needle seemed to penetrate the dura. About 15 cc. of the antiscarlatinal serum was given intraspinally, although but a few drops of blood-tinged fluid could be obtained.

On the 5th and 6th fifteen cc. of the serum were given, only a few drops of fluid being obtained each time.

On the 7th another unsuccessful attempt at cistern puncture was made, but a lumbar puncture, while the child was still under the anesthetic, yielded 8 cc. of yellowish, slightly cloudy fluid, and 15 cc. of serum was injected. This fluid was negative for organisms by smear and sterile by culture. The protein was somewhat higher than in the cisternal fluid. The sugar was normal by Fehling's. All this time the temperature had been running between 101° and 104° to 105°. Much of the time she had been irrational. The left ear had drained rather freely at times but there had been no indication of mastoid involvement, either clinically or by the roentgen ray examination. An examination of the eye grounds on February 1 showed some blurring of both discs but no peripapillary edema or chocking of the discs.

On February 8, the child seemed distinctly better. The neck was less retracted. She was rational and the temperature was lower—99.5° to 102.4°.

On February 11, an extensive serum rash developed, which was probably a factor in keeping up the temperature, which did not become normal until February 18. On the 11th a slight internal strabismus of the right eye was noted. This gradually disappeared.

From this time on her convalescence was fairly satisfactory. There were two exacerbations of fever, probably due to the lighting up of a pyelitis from which she had suffered some five years before and which had troubled her at intervals ever since.

Pus and albumin were found in the urine during one of these periods. Six other examinations of the urine were negative.

A blood count on February 2 showed 22,600 white blood cells, 83 per cent. polymorphonuclears, 11 per cent. lymphocytes, 6 per cent. mononuclears. Two blood cultures, one on the 2nd and one on the 20th of February, were negative.

The culture of hemolytic streptococcus from the spinal fluid was given to Dr. Anna W. Williams, who reported it as belonging to the chief scarlet fever group. It completely absorbed the antibody in serum produced by scarlet fever strain No. 55.

This identification of the causative organism seemed good reason for believing that the serum used was the specific factor in the recovery. The administration of the serum into the cistern was probably of great value, especially since the fluid withdrawn still contained living organisms. The development of block always renders the prognosis of a meningitis case much more serious. The only clinical features that could be referred to the adhesion were some loss of control of the sphincters and spasticity of the legs, more marked on the right side.

These manifestations gradually disappeared. The patient returned home in excellent condition on March 13, 1927. Her progress since that time has been entirely satisfactory.

Streptococci meningitis following scarlet fever is comparatively rare. We have a record of only four other cases. In all of these there was also an otitis media and in two of them a mastoid operation was performed. All resulted fatally. In this particular case it seems impossible to say whether the meningitis was secondary to the otitis media or whether both conditions developed more or less simultaneously and independently.

The high mortality of streptococci meningitis is shown by the fact that this is only the third case to recover among the 116 cases that have been seen by the Meningitis Division.

SPONTANEOUS MENINGEAL HEMORRHAGE

Published in The Journal of the American Medical Association, 1926,
Vol. LXXXVI, pp. 6-8

Josephine B. Neal

While the French have developed an extensive literature on spontaneous or medical meningeal hemorrhage, comparatively little has been written on the subject by the physicians of other nations. Aside from an article by Leopold, a chapter that the writer contributed to Tice's Practice of Medicine and a case reported by Dawson, there are but brief references to the subject in English. In Sweden, two comprehensive articles have been published by Ehrenberg and Forsheim, respectively. Only a few articles have been written on the subject in Germany. No attempt will be made to review the literature, the principal references only being given.

During the fifteen years that the members of the meningitis division of the Health Department of New York City have been studying acute conditions of the central nervous system, especially meningitis, poliomyelitis and epidemic encephalitis, we have seen over thirty cases, occurring for the most part in children and young adults, in which a diagnosis of meningeal hemorrhage was made. So few of the physicians with whom we have seen these cases have been familiar with the condition that it seems worth while to describe some of the more interesting cases in detail and to present certain data in regard to the others. These are listed in the accompanying table.

The early symptoms of the cases, especially when there was no underlying disease, were those of an acute, often of a fulminating, meningitis. The onset was sudden, with intense headache, usually vomiting, and not infrequently unconsciousness within a few hours. In some instances, there were twitchings or convulsions. Stiffness of the neck, a positive Kernig sign and various changes in the reflexes occurred within a few hours to a day or two. In most instances, there was a moderate elevation of temperature (from 1 to 3 degrees.) The pulse was usually, but not invariably, slow, especially early in the case. Ehrenberg, followed by Leopold, divides the cases into two types, meningitic and apoplectic. This has seemed unnecessary, as merely emphasizing a greater or less severity of onset which might easily be further subdivided. It has not seemed to influence the outcome, as several patients with early unconsciousness have made complete and, in some instances, fairly rapid recoveries.

In this group of cases none have been included except those noted below that showed spastic paralysis, as of course this would have led to the diagnosis of cerebral hemorrhage. In several instances there was a slight and transitory weakness of the muscles of the face or hand, probably due to pressure. Two cases with more marked paralysis came to necropsy and showed no evidence of cerebral hemorrhage.

Guillain states that the symptoms are due both to pressure and to the toxic products of hemolysis. The diagnosis has been made chiefly by the spinal fluids, which have contained varying amounts of blood in the different cases. The blood at a given puncture has been evenly distributed through the fluid, as shown by collection in successive tubes, and it has not coagulated. When the lumbar puncture was done very soon after the onset of symptoms, the supernatant fluid, on centrifugalization, was nearly clear, or slightly reddish or yellowish. In later fluids, the supernatant liquid was yellow, the depth of color depending on the amount of the hemorrhage. The spinal fluids have been sterile on culture, except in three instances in which the meningeal hemorrhage accompanied an epidemic meningitis. In many instances the Wassermann test has been done on the spinal fluid and has been negative (except in Case 22 in the table). The cytologic and chemical examination have not been of much value in most of the cases on account of the blood.

The following case history is fairly typical:

CASE 11.—M. W., a girl aged 12 years, whose past history was negative except for measles, was seen at midnight, Aug. 10, 1917. During the day she had received a slight blow on the head while playing with her brother. She had been taken suddenly ill at 2 p. m. with severe headache and vomiting, and began having periods of delirium. At midnight she was semistuporous; the pupils reacted sluggishly to light; the patellar reflexes, which had been exaggerated earlier, were nearly lost; there was stiffness of the neck and a positive Kernig sign; the temperature was normal, and the pulse 77 and slightly irregular. At lumbar puncture, 30 cc. of uniformly bloody fluid was withdrawn, and since the picture so strongly suggested meningitis and since horse serum is indicated in hemorrhage, 20 cc. of antimeningitis serum was given. On the following day the condition was slightly improved. The patient was less stuporous and more rational. At lumbar puncture 30 cc. of somewhat less hemorrhagic fluid was withdrawn and 20 cc. of serum again administered. On the 12th and 13th, no punctures were done, as the condition seemed to be improving. On the 13th she began to have a rather low, irregular fever. August 14 thirty cc. of bloody fluid was withdrawn but no serum was given, as the cultures from the previous fluids were sterile. The Wassermann test on the spinal fluid was negative. On the 15th another puncture was done, as the meningeal symptoms remained marked. The patient was sent to Bellevue Hospital, August 16, where she remained until the middle of October. The meningeal symptoms persisted for some time and necessitated repeated lumbar punctures, the earlier of which revealed hemorrhagic fluids. She complained of rather frequent headaches for some months after she left the hospital. When last seen, in the fall of 1924, seven years later, she showed no evidence of having suffered any ill effects from the meningeal hemorrhage.

A similar but much milder case is the following:

CASE 8.—G. K., a boy aged 9 years, with no history of a previous illness, was taken suddenly ill at 2 a. m., April 29, 1918, with headache, vomiting, and occasional periods of delirium. When examined, May 3, he showed moderate stiffness of the neck, marked Kernig sign, absence of the patellar reflexes, and contracted pupils. He was no longer delirious but was slightly stuporous. His pulse was 86 and irregular. He had had a low irregular fever. At lumbar puncture 15 cc. of hemorrhagic fluid was obtained and an equal amount of meningitis serum given, for the reasons already stated. The culture from the fluid was sterile and the Wassermann reaction negative. The boy made a rapid and uneventful recovery, and more than six years later was reported to be entirely well.

The following case was seen at the Jamaica Hospital through the kindness of Dr. E. E. Keet:

CASE 18.—J. S., a youth aged 19, had suffered from a severe electric shock a year previous to his illness and since that time had had frequent intense headaches, slight haziness of vision, twitching of the left hand, and slight left facial weakness. He was taken ill suddenly, Jan. 24, 1925, with headache, vomiting and early unconsciousness. He was seen, January 28. At this time there was moderate stiffness of the neck and Kernig sign. There was no limitation of motion of the eyes, no ptosis and no strabismus. The pupils reacted normally to light and in accommodation, and the fundi were normal. There was some facial weakness, more marked on the right. The patellar reflexes were diminished; there was no ankle clonus or Babinski sign, though the latter had been present on admission. The abdominal reflexes responded irregularly at different examinations. There was a rather generalized muscular tremor at times. The patient was still semistuporous. His speech was somewhat blurred. He had been having an irregular fever, up to 102. His pulse had varied from 50 to 90 but was regular in rhythm. The systolic blood pressure was 130; diastolic, 70. The blood count showed a moderate leukocytosis, 16,000. There was incontinence of urine. The lumbar punctures had yielded uniformly blood-tinged fluid. The Wassermann reaction on the spinal fluid was negative. The patient remained in the hospital for almost a month, gaining steadily. On leaving the hospital there was some weakness of the left hand, but this disappeared in about a month. The diagnosis of epidemic encephalitis was considered in this case, but meningeal hemorrhage was favored on account of the suddenness and severity of the onset. Moreover, hemorrhagic fluids are rare in epidemic encephalitis.

The following case was seen at Bellevue Hospital through the courtesy of Dr. Charles E. Nammack:

Case 25.—D. S., a woman aged 24, with a history of good general health, was not subject to headache until the two weeks preceding the illness. When the illness began, Oct. 4, 1924, she had been riding in a taxicab and had struck her head against the side of the cab, but there was no contusion of the scalp, and the roentgenogram of the skull showed no evidence of fracture. She became suddenly ill with epistaxis, vomiting, headache and pain in the back of the neck, and speedily became unconscious. The patient was examined October 7. At that time she was still quite stuporous; there was moderate stiffness of the neck and Kernig sign. The pupils were equal and reacted to light and in accommodation; there was bilateral ptosis and weakness of the left external rectus. The disks were choked. There was also slight facial weakness. The patellar reflex was increased on the left and diminished on the right. There was no ankle clonus. There was a positive Babinski sign on the left. There was no sensory disturbance. A low irregular fever ranged from 100 to 101°F. The pulse rate was from 60 to 70. The blood pressure was 120, systolic, and 80, diastolic. The blood count showed a low leukocytosis, 13,600; polymorphonuclears, 81 per cent. The urine was not abnormal. Chemical examination of the blood revealed: blood sugar, 140; nonprotein nitrogen, 34; sodium chlorid, 495. The blood and spinal fluid Wasserman reactions were negative. The spinal fluid was hemorrhagic. On account of the choking of the disks, a diagnosis of brain tumor was considered. However, the choking of the disks disappeared on repeated lumbar puncture, as did also the weakness of the face and eye muscles. The patient left the hospital greatly improved, and subsequent examinations have shown her to be entirely well after several months.

Case 10.—J. M., a girl aged 12, seen June 5, 1916, had had measles and scarlet fever some years before, and there was a history of an indefinite illness two months previously, with pain and swelling of the legs, and fever. She had been taken ill while at a dance late on the night of June 4, with headache and vomiting, followed very quickly by unconsciousness. June 5 she was semistuporous. There was stiffness of the neck and a positive Kernig sign. The patellar reflexes were absent; the pupils were unequal. The pulse was 84 and regular and the temperature, 100°F. At lumbar puncture, 35 cc. of hemorrhagic fluid was obtained, and 20 cc. of antimeningitis serum given. June 6 the patient was taken to Mount Sinai Hospital, where she died three days later.

Necropsy (by Dr. F .S. Mandelbaum).—The body was that of a poorly nourished young girl, showing marked rigor mortis and postmortem discoloration. There were no marks of injury on the body.

The skull was of normal thickness. The surface of the dura was normal in appearance. All the cranial blood sinuses were opened and found normal. Sphenoidal sinuses and both middle ears were opened and found normal. The internal surface of the dura was normal in appearance. The cerebrospinal fluid was normal in amount but, as it was mixed with blood from the necropsy, its characteristics could not be noted.

The brain was intensely congested and extremely bright red. Over its convexity were numerous small, bright red petechial hemorrhages. In the pia-arachnoid, at the base of the brain, especially over the pons, were great numbers of bright red, hemorrhagic areas from 2 to 6 mm. in size. The blood vessels were normal and contained fluid blood, but even the minute capillaries were congested and visible. Over the surface of the brain were two or three pale, miliary areas which had somewhat the appearance of tubercles. The brain was uniform in appearance and consistency. An attempt was made to aspirate the ventricles, but was unsuccessful. On section, the brain showed, throughout, the same extreme congestion noted on the surface; all the ventricles were normal, and the brain was otherwise normal.

Cultures from the brain showed *B. coli.* The smears were negative for tuberculosis. An inoculation, July 20, 1916, was negative for tuberculosis.

Smears with Gram's stain and diluted carbolfuchsin showed a moderate number of polymorphonuclears, but no organisms.

Microscopic examination of two sections of the cortex revealed a marked edema of the pia, and also a diffuse infiltration with red blood cells and few polymorphonuclears. There was a marked congestion of all the blood vessels of the pia and the brain. A moderate number of very small hemorrhages were found in the cortex. The hypophysis showed extreme congestion of the blood vessels and a small amount of colloid in some of the alveolar spaces in the pars intermedia and pars anterior. Acidophilic cells were very prominent and were present in great numbers; otherwise, the section was normal.

The Giemsa stain showed no bacteria.

In the cervical cord and pons there was congestion of the blood vessels and the same type of inflammation of the pia-arachnoid as noted in the meninges of the cerebrum. No evidence of poliomyelitis was found. The diagnosis was acute encephalitis.

It will be noted that it was assumed at the necropsy that the blood in the spinal fluid was accidental. While a diagnosis of acute encephalitis was made, it is evident from a study of the report that the changes were more marked in the meninges than in the brain substance.

Cases 13, 26 and 28 were of interest:

Case 13.—A girl aged 15 years, gave a history of a similar illness nine years before, which had lasted about three weeks. She had had another mild attack in 1915, and made an uneventful recovery. Two lumbar punctures were done, yielding 50 and 60 cc. respectively, of slightly hemorrhagic fluid.

Case 26.—A man aged 27, gave a history of a similar illness, not so severe, four years previously. His attack in 1918 was a severe one, with unconsciousness at the onset. The fluid was hemorrhagic at several punctures.

He made an apparently complete recovery but died in a similar attack some years later.

Case 28.—A man aged 30, seen in June, 1915, had had a similar attack eighteen years before, from which he had recovered completely, and a second attack five years before, which was followed by some mental impairment. The attack in 1915 was only moderately severe, and he recovered so far as his physical condition was concerned, but the mental impairment had apparently increased.

DATA IN THIRTY-FIVE CASES OF MENINGEAL HEMORRHAGE

No.	Year	Age	Sex*	Possible Cause	Outcome	Comment
1	1923	2 mo.	♀	Not determined	Died	
2	1917	5 mo.	♀	Epidemic meningitis	Died	
3	1918	9 mo.	♀	Not determined	Died	Liver enlarged; jaundice
4	1912	22 mo.	♂	Epidemic meningitis	Recovered	
5	1925	4 yr.	♀	Mitral lesion and Streptococcus viridans septicemia	Died	
6	1916	5 yr.	♀	Epidemic meningitis	Died	
7	1923	7½ yr.	♂	Hydrocephalus; mitral regurgitation	Died	
8	1918	9 yr.	♂	Not determined	Recovered	
9	1923	10 yr.	♀	Mitral stenosis	Recovered	
10	1916	12 yr.	♀	Not determined	Died	Necropsy finding reported
11	1917	12 yr.	♀	Not determined	Recovered	
12	1917	14 yr.	♂	Nephritis	Recovered	Died a month later with nephritis
13	1915	15 yr.	♀	Not determined	Recovered	History of similar illness 9 years before
14	1918	16 yr.	♂	Pneumonia	Recovered	
15	1919	17 yr.	♀	Not determined	Died	
16	1923	18 yr.	♂	Not determined	Died	Necropsy; pachymeningeal internal hemorrhage
17	1917	19 yr.	♀	Not determined	Recovered	
18	1925	19 yr.	♂	Not determined	Recovered	Severe electric shock year ago
19	1914	20 yr.	♂	Not determined	Died	
20	1924	20 yr.	♀	Not determined	Recovered	History of epidemic meningitis 10 years ago
21	1918	20 yr.	♀	Epidemic meningitis	Recovered	
22	1917	21 yr.	♂	Syphilis	Recovered	Sent to State Hospital
23	1915	22 yr.	♂	Blood pressure, 190 systolic	Died	No focal symptoms pointing to cerebral hemorrhage
24	1914	22 yr.	♀	Endocarditis	Died	
25	1924	24 yr.	♀	Struck head in taxicab, but no external mark	Recovered	

* In this column, ♂ indicates male; ♀, female.

DATA IN THIRTY-FIVE CASES OF MENINGEAL HEMORRHAGE—*Continued*

No.	Year	Age	Sex*	Possible Cause	Outcome	Comment
26	1918	27 yr.	♂	Not determined	Recovered	History of previous attack 4 years ago; died in similar attack some years later
27	1924	27 yr.	♂	Not determined	Recovered	
28	1915	30 yr	♂	Not determined	Recovered	History of 2 previous attacks; mental deterioration after second and third
29	1917	34 yr.	♀	Not determined	Recovered	
30	1919	36 yr.	♂	Not determined	Recovered	
31	1925	36 yr.	♀	Not determined	Died	Necropsy showed hemorrhage in right ventricle
32	1920	40 yr.	♀	Not determined	Recovered	
33	1923	46 yr.	♂	Not determined	Recovered	
34	1923	48 yr.	♂	Not determined	Died	Necropsy showed hemorrhage in left ventricle and around circle of Willis
35	1923	50 yr.	♀	Not determined	Recovered	

* In this column, ♂ indicates male; ♀, female.

While realizing that it is possible that these other attacks were different in origin, it seems reasonable to assume that they were of the same nature. If they were, the possibility suggests itself that they represented stages in the development of pachymeningitis interna hemorrhagica. It will be recalled that, on necropsy in this condition, there are often several layers in the false membrane beneath the dura, showing successive episodes in its formation.

It will be noted in the table that in four cases the meningeal hemorrhage accompanied an attack of epidemic meningitis; in four cases, a cardiac lesion; in one, pneumonia; in one, syphilis; in one, nephritis, and in one, high blood pressure. In the great majority of cases, however, no cause could be determined. Froin gives a long list of conditions that may be the underlying causes of spontaneous meningeal hemorrhages. The author stated in Tice's Practice of Medicine: "When we see so many conditions given as the cause of an occurrence, and when we remember that these conditions exist in the vast majority of cases without this occurrence taking place, it seems evident that the real cause must lie elsewhere, perhaps in some inherent weakness of the blood vessel in the individual cases in which meningeal hemorrhage occurs." Meylahn concludes that spontaneous meningeal hemorrhage is nearly always a diapedesis. The causes of such an occurrence are disorders in the function of the vascular nerve apparatus. These vasomotor disturbances can be the result of diseases of the blood vessel walls. In that case, however, there must be assumed a previously existing constitutional weakness of the vascular nerves.

Weil, Isch-Wall and Pollet call attention to the existence of blood lesions, analogous to those of hemorrhagic conditions, existing in certain patients with meningeal or cerebral hemorrhage. There is an increased bleeding time; coagulation is retarded, and the clot is less retractile and easily dissolves. The importance of this from a therapeutic standpoint is obvious.

The most important measure in treatment is lumbar puncture. Guillain warns against withdrawing too much fluid early in the illness. The lumbar puncture should be repeated daily, or less often, depending on the clinical symptoms. In most of our cases it was followed by improvement. In many of our cases horse serum, in the form of antimeningitis serum, was injected intraspinally early. This may have had a favorable influence on the hemorrhage. Certainly when the bleeding and coagulation time are increased, measures should be taken to correct these conditions of the blood.

It will be seen from a study of the table that twenty-one of our thirty-five patients recovered from the meningeal hemorrhage, although one died of nephritis a month later.

Meningeal hemorrhage is certainly a most interesting condition and one that warrants much further study.

For bibliography see the original article.

EXPERIMENTAL POLIOMYELITIS

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Mary Nevin and Florence R. Bittman

The object of this work was to repeat certain experiments on poliomyelitis in rabbits. A number of workers have reported varying results. Some have found it difficult or impossible to inoculate rabbits and produce a picture resembling poliomyelitis; others assert that they were able to make successful inoculations and to pass the virus through a number of generations. All who were successful have found, however, that it was only in young rabbits that any effects were produced; that the results were variable and that the symptoms were always different from those observed in monkeys and in man.

The most recent work on experimental production of poliomyelitis in rabbits is that of Rosenau and Havens. They passed poliomyelitis virus from a monkey through eight generations of rabbits. The virus showed no signs of dying out. It gave no evidence of becoming more virulent. The incubation period remained variable, and the percentage of positives did not increase. All inoculations were by no means successful, and there was great individual difference in susceptibility. Only 40% of the animals succumbed. These authors describe two types of symptoms. In one type, designated as the "progressive," there was paralysis of one extremity progressing until death occurred, resembling somewhat the type of disease found in monkeys and man. In the other group, designated as the "fulminating" type, the symptoms were more explosive in character with extreme weakness and prostration, death coming on within a few hours as a result of respiratory failure. The period of incubation was variable. It did not depend on the route of inoculation. The pathologic changes were different from those in monkey and man so far as microscopic examinations were concerned. "They lacked the distinctive features of the pathological picture of poliomyelitis in man and the monkey. Capillary congestion, punctate hemorrhages, degeneration of the motor cells, satellitosis and more or less cellular infiltration of the gray matter of the cord and medulla are found, but perivascular infiltration is absent and the infiltrating cells are not lymphocytic in character." These authors feel, however, that they were dealing with a modified form of poliomyelitis; that the rabbit reacts differently to the virus than do monkeys and man. They raise the question, therefore, whether poliomyelitis may not occur in other animals in some hitherto unrecognized or unnoticed form.

Krause and Meinicke, and Lenz and Huntemüller had previously reported the successful passage of poliomyelitis virus from rabbit to rabbit through successive generations. They found only slight lesions in the brain and spinal cord. These workers obtained better results by intravenous and intraperitoneal inoculations than by the intracerebral route.

Failure to inoculate rabbits successfully has been reported by Römer and Joseph, Landsteiner and Levaditi, Leiner and von Wisner, and by Flexner and Lewis.

Interesting results were obtained by Marks, who was able to pass poliomyelitis virus through 7 generations of rabbits. The effects in rabbits, however, were not definite. Death occurred in some of these animals in from 7 to 11 days, with development of motor weakness and convulsions just preceding death. Monkeys inoculated with brain suspensions of these experimental rabbits developed similar symptoms. The pathologic changes noted in the rabbits were not characteristic, the only findings being hyperemia of the brain. As Marks states: "The disease thus produced in rabbits cannot be recognized as poliomyelitis." He finds further that "not all strains of the virus can be transmitted successfully to even a small fraction of all varieties of domesticated rabbits."

A 5% unfiltered suspension of poliomyelitis virus was used. This had been obtained from the Rockefeller Institute. Inoculations were made intracerebrally, intraperitoneally and intravenously. The intracerebral route was the one most frequently employed. In some animals the intracerebal route was combined with the intravenous or with the intraperitoneal route. Only young rabbits were used; the average age was 6 weeks.

First Passage.—Ten rabbits, 344 to 353, were inoculated intracerebrally with from 0.25 cc. to 2 cc. At the same time monkey X was inoculated intracerebrally with 0.6 cc. of the same virus. Only one rabbit, 348, developed typical symptoms (6th day); the animal was chloroformed on the 13th day. One rabbit was discharged after 10 weeks, and the remaining rabbits died after from 24 hours to 1 month. Monkey X, used as a control, developed typical symptoms of poliomyelitis on the 5th day and died on the 9th day. Examination showed the changes characteristic of poliomyelitis.

Second Passage.—Pooled brain suspension from 2 rabbits (348 and 350) of the first passage was inoculated into 2 rabbits, 358 and 359, both intracerebrally and intravenously. One of the rabbits (348) furnishing this virus, it will be recalled, was the one which had developed typical, progressive, paralytic symptoms, and the other (350) had been found dead after 7 days.

Brain suspension of monkey X was also inoculated in a second series of 4 rabbits, 354 to 357, (2d passage of virus). The doses varied from 1 cc. to 1.5 cc. The intracerebral route alone was used in this group. None of these animals developed any symptoms.

Third Passage.—The pooled brain suspension from rabbits 355 and 356 was inoculated into a series of 4 rabbits (372 to 375). One of the rabbits (374) developed paralytic symptoms on the 14th day. The symptoms were similar to those observed in rabbit 348 of the 1st passage. Suspension from the brain and cord of rabbit 356 was also inoculated in a 2d set of rabbits (376 and 377), both intracerebrally and intraperitoneally in the one, and intracerebrally alone in the other. No symptoms developed in these animals.

Fourth Passage.—A suspension from the brain of rabbit 374 of the 3rd passage of virus was next inoculated in a series of 10 rabbits (378 to 387). The doses from the intracerebral inoculations varied from 0.25 cc. to 0.5 cc., and all rabbits of this series except one were also inoculated intraperitoneally with 2 cc. of the suspension. All of these rabbits died in from 15 minutes to 4 days. No symptom developed.

Fifth Passage.—A pooled brain and cord suspension from rabbits 378, 379, 382, 383, and 387 was inoculated in a series of 4 monkeys (93, 52, 53 and 31) and 6 rabbits (394 to 399). The monkeys were given 1 cc. of suspension intracerebrally and from 3 to 20 cc. intraperitoneally. The rabbits were given 0.4 and 0.5 intracerebrally and 5 cc. intraperitoneally. In none of the animals of this series did any symptoms develop. The virus had apparently died out.

Pathologic Changes.—Tissues of rabbits 350, 351 and 353, examined by Christine I. Gruggel, showed a slight amount of neurophagocytosis of the ganglion cells and some cell degeneration. There was no evidence of round cell perivascular infiltration. As the degeneration of the ganglion cells might be due to other causes, there was no conclusive evidence of poliomyelitis, but it cannot be excluded.

In rabbits 344, 345, 348, 373, 374, 375, 377, 395 the meninges were normal; in the gray matter occasional ganglion cells showed cytolysis and neurophagocytosis, the latter being mononuclear. There was no cellular infiltration or evidence of poliomyelitis.

Sections of monkey X showed a round cell infiltration of the meninges following the vesels into the gray matter of the anterior horns. In places were patches of exudate in which was degeneration of ganglion cells, with neurophagocytosis—a typical picture of poliomyelitis.

Discussion

In only 2 rabbits did symptoms occur that were at all comparable to those in poliomyelitis of monkeys and of man or that corresponded with those described by Rosenau and Havens in their "progressive paralytic" type. One of these rabbits (348) of the 1st passage of virus developed symptoms after an incubation period of 6 days. There was weakness of the posterior extremities, which were held wide apart. There was definite paralysis of the left foreleg and paresis of the right foreleg. This animal was chloroformed on the 13th day. Necropsy did not show any definite lesions, and the pathologic examinations showed only some cytolysis of the ganglion cells and mononuclear neurophagocytosis. There was no cellular infiltration. Cultures to determine the presence of aerobic and anaerobic micro-organisms were all negative. Rabbit 374 of the 3rd passage of virus developed similar symptoms on the 14th day. This rabbit had been inoculated with virus passed through monkey X, used as a control in the first passage; the changes were the same as in rabbit 348.

Of the remaining 26 rabbits which succumbed, 5 died within 24 hours or less. These must be left out of consideration, as death may have been due to injury or shock. In the case of 3 rabbits dying almost immediately, anaphylactic shock due to a foreign protein may have been the cause. In the case of 21 rabbits surviving the first 24 hours and living in some cases as long as a month, it is impossible to determine the cause. Some of them exhibited the symptons described by Rosenau and Havens as of the "fulminating" type. Some of them were found dead without any symptoms having been noted. In the necropsis and examinations of tissue sections of these animals, no changes characteristic of poliomyelitis were found; at least there was an absence of such cnanges as are found in poliomyelitis of monkeys and man. The dose and the route of inoculation does not seem to have caused any difference in the effect. The period of incubation was variable in all passages. There was apparently no change in the virulence of the virus.

The question of anaphylactic shock was carefully considered in carrying out these experiments. A series of 6 rabbits, 338 to 393, were inoculated with 5% suspension of normal rabbit's brain. None of these animals developed any symtoms, and they were discharged after a period of 2 months. This may, however, be merely a coincidence. It may be that none of the animals in this small group of controls were sensitive to the brain protein of the particular group of animals used.

It is difficult to make any definite deductions from the results of the foregoing investigation. If the rabbits exhibiting the "fulminating" type of symptoms died as a result of poliomyelitis virus, we should be forced to accept the suggested hypothesis of Rosenau and Havens that in some species of animals, poliomyelitis develops or assumes a type of disease hitherto unrecognized or unnoticed. Even in the 2 rabbits which developed symptoms similar to those occurring in poliomyelitis of monkeys and of man, the histologic changes were not characteristic. As, however, in both types of effects certain histologic changes were present, it cannot be denied absolutely that they were caused by poliomyelitis virus. The changes present, although different from those found in monkeys and man, may nevertheless be the changes produced by poliomyelitis in rabbits. The fact that one of the rabbits developed typical symptoms from virus that had been passed through a monkey and one generation of rabbits, would certainly suggest such a possibility. The symptoms and pathologic changes were the same as in the case of the rabbit of the first passage inoculated directly with fixed virus.

SUMMARY

In the experiments on young rabbits, symptoms resembling poliomyelitis were observed in a small number. A larger number of rabbits died after varying periods of incubation, but without showing characteristic symptoms of poliomyelitis. The virus did not show any increasing virulence by passage through rabbits. In one series of animals, the virus apparently died out after one passage. In the other series, it apparently died out after passage through a monkey and 3 sets of rabbits. Brain suspension from this 3rd passage through rabbits, inoculated in both monkeys and rabbits, failed to have any effect. In the case of the animals which died, the factor of infection was carefully controlled by cultures; also the possibility of anaphylactic shock due to a foreign protein was taken into account by inoculating a series of young rabbits with normal rabbit brain suspension. All of these controls were negative. It cannot be said that we have been able successfully to inoculate rabbits with poliomyelitis.

For bibliography see original article.

THE RELATIONSHIP BETWEEN DIFFERENT ANTIBODIES OF HEMOLYTIC STREPTOCOCCI FROM SCARLET FEVER.

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Anna W. Williams, Caroline R. Gurley, et al.

Studies on strains of hemolytic streptococci from different cases of scarlet fever, from normal throats and from cases of other diseases are being carried on by workers in our laboratory along three main lines:

1. Grouping by agglutination, and absorption of agglutinins. In these studies we have been assisted by H. D. Hussey, F. Bittman, F. Berman, A. Brassel and others.
2. Grouping according to the protective power of serum stimulated by the inoculations of whole cultures. This part of these studies has been carried on mainly by C. R. Gurley, M. F. Carmichael and F. Berman.
3. Grouping according to the power to produce toxic filtrates neutralized by convalescent scarlet fever serum, and by serum from horses inoculated with a toxic filtrate. We have been assisted in this work by H. D. Hussey, A. Brassel, B. Kolchin and others. Most of the human inoculations were made by A. Zingher and R. G. Freeman.

The main purpose of all of these studies has been to determine the relationship of hemolytic streptococci to scarlet fever and to so-called natural immunity from scarlet fever.

In regard to grouping by agglutination, we have found that out of about 70 strains tested, only about 30 per cent. fall into one group; 7 per cent. consist of two small groups, one of 3 strains and one of 2 strains; about 63 per cent. do not fall into these groups.

The strain of Dochez, Avery and Lancefield "No. 273" which they first listed in their work on agglutination[1] as coming from a case of scarlet fever belongs, according to the absorption test, to our heterogeneous group, but the strain Dochez gave to Dr. Huntoon, who kindly sent it to us, labelled "No. 1372", falls by the absorption test into our dominant group—which we have called Type I group. Two strains kindly sent us by the Drs. Dick labelled "No. 1" and "No. 2" both fall into this Type I group.

Eight per cent. of strains from sources other than scarlet fever fall into our Type I group—2 in 25. One of these was from a wound and the other from an endocarditis case.

In view of the fact that only about 30 per cent. of our strains from scarlet fever fall into one group, we conclude that the agglutination test, whatever the significance of the occurrence of such an agglutination group among scarlet fever strains, is not of much practical worth in the diagnosis of scarlet fever or in the identification of carriers.

In regard to grouping according to their power to stimulate the production of antibodies protective in mice against whole cultures, all of our strains from scarlet fever so far tested and our 2 strains from sources other than scarlet fever fall into one group.

We have not studied by this test many of the strains studied in our agglutination work, chiefly because of the difficulty in raising the virulence of some strains, but we have tested enough strains to demonstrate that this power to stimulate the production of protective antibodies is probably broader than the power to stimulate agglutinins.

Thus, while our two strains F and K from other sources, one from a wound and one from endocarditis, are controlled by our Type I serum, one of our measles strains M 22—which falls into our heterogeneous agglutination group is controlled as well. We have so far raised the virulence of 19 strains to a ten millionth of a cubic centimeter or less as a lethal dose for mice—13 from scarlet fever and 6 from other sources.

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[1] *Exper. Med.*, 1919, 29, 215.

In regard to the third line of these studies—studies on toxic filtrates—we have tested many strains of hemolytic streptococci for their power to produce neutralizable toxic filtrates.

We have found that not only do all of our scarlet fever strains tested, which were isolated over 3 years ago, produce such a toxic filtrate, but all pyogenes strains isolated from a very recent series of scarlet fever cases do the same.

Our two strains from sources other than scarlet fever that fall into our protective group, and therefore into our agglutination group, do the same. Also 2 strains that are not protected by our Type I serum and that do not fall into our agglutination group—one from osteomyelitis and one from bronchitis produce neutralizable toxic filtrates.

Some strains from sources other than scarlatina, but which belong to the pyogenes group, and a few outside of the pyogenes group have either not produced toxic filtrates or have produced toxic filtrates that are not neutralized by scarlet fever convalescent serum or by our horse serum.

We have examined a series of 25 cases of excised tonsils with a history of no recent exposure to scarlet fever. From 56 per cent. of these (14 cases) hemolytic streptococci were isolated, and 42.8 per cent. of these (6 cases) produce toxic filtrates neutralized by convalescent scarlet fever serum. These strains have not yet been tested by agglutination or by mouse protection.

In this connection it is interesting and significant to report on our comparative results in protection in mice against the living culture with serum from horses inoculated with toxic filtrates, and from those inoculated with whole cultures of these streptococci from scarlet fever. Our whole culture streptococcus serum affords marked protection in mice against all of our strains protected by our Type I rabbit serum, while our toxic filtrate horse serums, which are yielding a serum having a high neutralizing value, do not yet protect at all.

The serum from Dochez' first bleedings that he sent us do not protect either, but Dochez now states[2] that he has a serum that protects against the whole culture and neutralizes the toxic filtrate as well.

These facts add to the evidence in favor of certain hemolytic streptococci being the cause of "scarlet fever."

But they point toward the probability of "scarlet fever" being only one of the manifestations of infection by a large group of these cocci.

They suggest that, while immunizing with toxic filtrates may prevent clinical scarlet fever, it may not prevent the more serious effects of hemolytic streptococcus infections. They also suggest that serum stimulated by the inoculations of toxic filtrates alone may not be so effective a curative agent as one stimulated by the simultaneous inoculations of toxic filtrates and whole cultures of the streptococci.

They finally raise the interesting question as to the possibility of there being strains of hemolytic streptococci in the throats of many people which, while not causing clinical scarlet fever, may produce immunity from such a condition.

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[2] Personal communication.

SCARLET FEVER

Etiology, Prevention by Immunization, and Antitoxic Treatment*

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William H. Park, et al.

The etiology of scarlet fever has been under active investigation for many years. Loeffler, in 1884, and later other bacteriologists noted that the hemolytic streptococci were very abundant in the throats of acutely ill scarlet fever patients. Clinicians also noted the fact that in certain cases of wound infection and puerperal fever a scarlet fever-like rash developed. These observations led to the opinion that there was a close relationship between certain strains of hemolytic streptococci and scarlet fever. The majority, however, held that the hemolytic streptococci were merely very important secondary invaders. Repeated attempts by numerous investigators to produce any disease similar to scarlet fever in animals by the inoculation of throat secretions of cultures of hemolytic streptococci obtained from the throats of scarlet fever patients failed. It was therefore necessary to turn to tests on man.

Moser reasoned that, if the streptococcus was the cause of scarlet fever, a curative serum might be produced, and in 1902 published a report of the good therapeutic results obtained by the use of an antiscarlet fever serum produced by the horse after frequently repeated injections had been made of living streptococci together with the broth in which they had developed, so as to get the antigenic value of the organisms as well as of any toxins that might have developed in the broth. The strains of streptococcus used were obtained from a number of toxic cases of scarlet fever.

A Russian investigator, Savchenko, went a step further; he showed, in 1905, that the serum contained both scarlet fever antitoxin and streptococcic bactericidal bodies. He also proved that the filtered broth in which the culture had grown contained a strong toxin. He said:

"Immunizing with the microbes only, it is possible to obtain a very active antimicrobal serum, but not at all antitoxic; vice versa, by immunizing with a toxin, we obtain an antitoxic serum and, therefore, one not acting on the microbes.

In scarlet fever, in which we have severe local infections and at times even general infections together with the toxemia, we must have in the serum antitoxins as well as germicidal antibodies. Filtrates of our own cultures of streptococci from scarlet fever grown in different combinations of broth proved to be nonpathogenic for rabbits, even in large doses. To conclude from this that there is no poison in the medium would be just as erroneous as to infer that there is no tetanus toxin after we had tried it on chickens, which are insusceptible to it.

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* This study of scarlet fever was aided by a grant of money from the Milbank Memorial Foundation.

And yet Moser, using broth cultures, obtained a serum which unquestionably acted on human beings antitoxically. It is obvious that either the streptococci produced a toxin in the medium used by Moser, or the toxin developed in the animal's system after its infection with living bacteria. In the latter case the method of immunization would have to be changed in the direction of the greatest possible increase of the number of bacteria in the material to be injected.

Therefore, before definitely choosing the kind of medium and the method of immunization, we made a few experiments with filtrates on the same horses that were subsequently used for immunization.

Martin's broth, which is being used for diphtheria toxin, was used as a culture medium in this experiment. The growth may be improved by adding to the broth either 0.5 per cent. dextrose or 2 per cent. ascitic fluid. Though the addition of sugar increases the growth of the streptococci considerably more than does the addition of ascitic fluid, it (by the products of fermentation) changes the reaction of the culture, making it acid. It should be expected, as is the case with diphtheria toxin, that this will prevent the formation of toxin. Indeed, an experiment on horses shows that filtrates of cultures with sugar are very little toxic, whereas filtrates of broth cultures with ascitic fluid clearly showed toxicity:

Two horses were injected with 15 cc. of filtrates of four days' broth cultures in the following order: Horse 1, fifteen cc. filtrate of broth sugar culture containing an abundant number of streptococci; Horse 2, fifteen cc. filtrate of broth ascitic culture containing a moderate number of streptococci. After twenty-four hours, only a faintly noticeable swelling was seen at the site of inoculation of Horse 1, and no rise of temperature was observed. Horse 2 developed quite a considerable edema and a rise of temperature. When the reaction from inoculation in Horse 2 disappeared, the injection of the same amounts of the same filtrates were repeated, but in a reversed order. Horse 1 developed a very strong reaction, whereas Horse 2 did not react at all.

It is obvious that cultures grown on 2 per cent. ascitic broth are preferable for toxin production to those grown on sugar broth, even if the organisms are less luxuriant.

The same filtrate, injected under the skin of rabbits in amounts of 5 and even 10 cc., produced no symptoms except a slight swelling. The filtrate turned out to be just as harmless for other laboratory animals.

These experiments showed that it is possible to obtain a sufficiently strong toxin, but they unfortunately also showed that by means of our laboratory animals it is impossible to gage either the amount of toxin—which is so important for proper immunization—or the amount of antitoxic substances in the serum, which is not less important for curative purposes. For the time being we are compelled to work, so to speak, by "feeling" our way.

As long as the broth cultures were shown to contain toxin, it appeared most proper to immunize horses with living cultures with the accompanying toxic broth, because in this way both antitoxic and antimicrobal substances should be obtained."

After reading the paper of Savchenko, no one can doubt that he produced by the filtrate a true antitoxic serum.

HUMAN IMMUNIZATION AGAINST SCARLET FEVER AND VALUE OF THIS EVIDENCE IN FAVOR OF THE CAUSAL RELATIONSHIP OF STRAINS OF STREPTOCOCCI TO SCARLET FEVER.

Gabritschewsky had finished an experimental study on a streptococcic infection of horses in which an appreciable rash developed, and believed he had proved that a vaccine of toxin and dead streptococci was able to develop a moderate degree of immunity. He then turned to the question of utilizing a streptococcus vaccine made from the toxin and cells of streptococci obtained from scarlet fever. His vaccine was made of bouillon, in which the streptococci had grown for four days, plus the streptococci themselves. The toxic bouillon containing 3 per cent. by volume of the streptococci was heated to 60 C. to kill the streptococci, and then preserved with 0.5 per cent. phenol (carbolic acid). Three doses were given subcutaneously one week apart. For infants under 2 years the dose was 0.25 cc.; for children between 2 and 10 years, 0.5 cc., and for those over 10 years, 1 cc. The results in children were most important. Langowoi, using his vaccine, found that of 120 childen inoculated, 13.3 per cent. developed a fine scarlet fever-like exanthem which was not followed by desquamation. A few developed a sore throat and strawberry tongue, and fewer still vomited. He states that these symptoms are those which are characteristic of scarlet fever, and these results of the vaccination add an extremely important proof "for the specificity of a scarlet fever streptococcus and its toxin." There is no question that Gabritschewsky considered the bouillon to contain a true toxin, not only from his language, but also because he was in contact with Savchenko, and their articles appear together a little later in the same Russian journal. He found that the second and third injections, although they were twice and four times as large as the first, rarely produced a rash. This he considered as evidence of the rapid development of immunity.

Gabritschewsky, in 1907, gives further evidence in favor of vaccination. Of 248 children receiving injections of the vaccine, Nikitin noted the rash in 17.3 per cent. In six he noted a generalized rash, vomiting, a strawberry tongue and an angina. In seven cases, the lymph glands were slightly swollen; in ten cases, albumin appeared in the urine for a day. In one case there was an acute nephritis, which ended in recovery. Twenty-six children who had previously had scarlet fever showed either no reaction or only a slight local swelling. A group of children tested during their fourth week of convalescence showed a greater percentage of reactions than those who had been convalescent for a long time. At Gabritschewsky's suggestion, Langowoi vaccinated twenty children with a vaccine made in the same way as the scarlet fever vaccine from the streptococcus of erysipelas. None of these developed a rash. These case are too few to prove that the result would have been the same if a larger number had been vaccinated, but they are suggestive of the specific character of the scarlet fever toxin. Gabritschewsky closes his article with five conclusions, of which I quote the last two:

"The fact that a scarlet streptococcus vaccine (toxin and culture) is able to produce a scarlatiniform rash and the other symptoms of scarlet fever is a decisive proof in favor of accepting a streptococcus as the specific cause of scarlet fever.

This decisive proof adds a scientific reason for the use of Moser's serum and my vaccine."

Gabritschewsky's death prevented him from following up the practical results of his vaccine. The following statement by Polotevkova is one of several encouraging reports:

"A severe outbreak of scarlet fever took place in my district, with 1,200 cases of the disease. Not only children, but also adults between 20 and 50 years of age were among the sick. The form of the disease was also severe, very often involving suppurative affections of the mouth, nose and upper respiratory tract. Owing to the extreme poverty of the peasants of this district, where very often milk could not be obtained for the sick, the only hope was in the preventive immunization. In short, the results were such that in the villages in which vaccination took place only one or two or no cases at all were observed; in villages in which no vaccination was carried on, the number of sick would reach from six to 175 cases. The author vaccinated her own three children."

Gabritschewsky considered that he had added to the proof already gathered sufficient evidence so that scarlet fever could be considered to be in the same class as diphtheria. The scarlet fever streptococcus produced in toxins in the throat. The absorbed toxins produced the rash, the fever and the other symptoms due to toxemia. The lowered resistance allowed the scarlet fever streptococci or other streptococci to invade distant parts and make the so-called complications. Additional evidence of the casual relationship was the development of a case of scarlet fever by the accidental inoculation in our laboratory of the throat of one of the assistants by a culture of several strains of streptococci including a scarlet fever strain. A typical case of scarlet fever developed, but attempts to reproduce the disease in animals with the culture failed.

In 1918, Schultz and Charlton described the reaction known by their name. When 1 cc. of a diluted convalescent serum is injected intracutaneously into the reddened skin of a scarlet fever patient, a blanching occurs in an area several centimeters in diameter. In December, 1923, Mair published further results and concluded that this reaction is due to the action of an antitoxin on the scarlet fever specific toxin. This brings us to the discoveries of the Dicks and of Dochez. In spite of the proof, which was largely unknown to most workers, the majority still believed that the hemolytic streptococcus found in scarlet fever was only an important complicating infection. In October, 1923, the Dicks inoculated five volunteers, who had lived in the country, with a streptococcus culture from a case of scarlet fever. One developed a typical case of moderate scarlet fever. The throats of five others were inoculated with the filtrate of a broth culture. None of these developed the disease, but when the same persons were inoculated with the streptococcus, one developed it. This proved that the infection agent was not a filtrable virus attached to the streptococci. It was found by them that the streptococci isolated by them from scarlet fever differed from one another in the ability to ferment mannite. The first two cases had been caused by an organism that fermented mannite. The Dicks then tried out the effect in the human skin of the toxin discovered by Gabritschewsky and Savchencko. They found that weak solutions of the toxin may be employed in skin tests to determine susceptibility to scarlet fever. The toxin is first carefully standardized and diluted so that 0.1 cc. represents a skin test dose. The test consists of an intradermal injection of exactly 0.1 cc. of the skin test dilution on the flexor surface of the forearm. The reaction is observed at the end of twenty-four hours. An area of reddening 2 cm. in diameter indicates marked susceptibility; 1 cm. in diameter, some degree of susceptibility to scarlet fever.

In a series of skin tests, reported in January, 1923, the Dicks found a positive or strongly positive reaction in 41.6 per cent. of the persons who gave no history of scarlet fever, and negative or only slightly positive reactions in all the convalescent fever patients tested. Zingher's observations fully corroborate these findings and show that the results in age groups from the Dick test were very similar to the results from the Schick test.

This skin test was developed before they attempted to produce experimental scarlet fever with the type of streptococcus that does not ferment mannite. In selecting their volunteers for inoculation with this type of streptococcus, they chose one with a negative skin reaction and one with a positive reaction. The two volunteers were inoculated with the same culture. The one with a negative skin test remained well, while the volunteer who had shown a positive test developed scarlet fever.

In the instances in which they had an opportunity to observe the skin reaction before and after an attack of scarlet fever, it was positive before the attack, and negative during convalescence. It is now known that there are about 10 per cent. of exceptions to that rule.

These authors also found that if persons with positive skin tests received convalescent scarlet fever serum intramuscularly, their skin tests became negative. With more definite doses than Gabritschewsky used they then attempted to immunize persons against scarlet fever and utilize later their skin test to determine the development of antitoxic immunity. They found that, by proper dosage, they were able to immunize persons with positive skin tests so that their skin tests became negative and they did not contract scarlet fever on exposure.

They report a series of 125 persons exposed to scarlet fever. In this series, sixty-three showed negative skin reactions and were not immunized. Fifty-two had positive skin tests, and were immunized with toxin. None of these persons contracted scarlet fever. They emphasize the importance of carrying the immunization to the point of a negative skin test. By the employment of three graded doses of toxin, they have been able to eliminate severe reactions.

While the Dicks were adding to the proof as to the causal relationship of certain strains of streptococci to scarlet fever, Dochez was developing an antitoxic serum through a method, developed by himself, of injecting subcutaneously into animals, masses of nutrient agar infected with scarlet fever streptococci. These injections, repeated from time to time in increasing amounts during from six to eight months, were found to develop a potent antitoxin. The serum of horse that had been subjected to such injections was found to give the Schultz-Charlton test, even when the serum had been diluted several thousand times. The serum was used in cases in New Haven and in New York, and the same results were regularly obtained as had been formerly obtained with unstandardized serum by Moser and Savchenko; but now the potency of the serum was established by the striking results with the Schultz-Charlton reaction. These were a demonstration to all that antitoxin was truly present. The interest developed by Dochez's announcement caused a number of biologic plants to use his methods, while others utilized toxin standardized by the Dick test, or crude toxic broth and dead organisms after the manner of Moser and Savchenko.

Shortly after Dochez announced his serum, the Dicks made a report on the production of antitoxin by the toxic filtrate standardized by their method. Whether the Dochez method or the various modifications of the toxin with or without killed or living streptococci will finally be adopted remains for further comparative tests to decide. This will depend largely on the value of an antimicrobal substance in the serum. The results with the Dochez serum or the Dick serum agree absolutely with the reports of the use of the Moser serum. The Dicks have suggested that the antitoxin be standardized by the number of skin doses it neutralizes. The federal government has accepted this, and a tentative unit has been adopted by antitoxin producers, but not yet accepted by the Dicks. A unit is defined as the amount of antitoxin that is required to neutralize 50 skin test doses to the extent of preventing a reaction of one cm. in diameter of scarlet fever toxin. This is determined by the method suggested by the Dicks of mixing 2 cc. of a 1:10 dilution of serum with 2 cc. of a dilution of standard toxin (200 skin doses) and injecting 0.1 cc. At the same time a control Dick test and a control serum test are made, to rule out protein and serum reactions. If the patient gives a good Dick test, no serum reaction and no other non-specific protein reaction, 1 cc. will neutralize at least 2,000 skin doses and contains at least 20 units. If neutralization occurs, higher dilutions of serum are used for further tests.

ACTIVE IMMUNIZATION

Earlier in this paper the Author gave the work of Gabritschewsky and his followers. In the light of the Dicks' researches and the rashes that developed, it can be estimated that the Russian infants received about 2,000; 4,000 and 8,000 skin test doses and older children 4,000; 8,000 and 16,000, or

TABLE 1.

IMMUNIZING RESULTS OF THREE INJECTIONS OF 300, 500 and 1,000 SKIN TEST DOSES OF SCARLET FEVER TOXIN GIVEN AT INTERVALS OF ONE WEEK

	Results of Retest	
Before Injection Reaction to Dick Test	Two Weeks After Last Injection	Four Weeks After First Injection
	2=2+	
	2=$+^1$	50 per cent. immune
10 + + or + $+^1$	1=+	
(Strong reactors)	5=−	50 per cent. still susceptible
	4=+	68.5 per cent. immune
35 $+^1$ or +	7=±	20 per cent. doubtful
(Moderate reactors)	24=−	11.5 per cent. probably susceptible

RESULTS WITH INJECTIONS OF DOUBLE THE FOREGOING AMOUNTS

Before Injection	Two Weeks After First Injection	Four Weeks After First and Two Weeks After Third Injections
12 + + or + $+^1$	3=2+	2=$+^1$
(Strong reactors)	3=+Week	1=±
	2=±	9=−
	3=−	
	1 not tested	
	27 per cent. immune	75 per cent. immune, 16 per cent. not immune, 8 per cent. doubtful

++ Indicates a Dick reaction of 2 cm. in diameter and + of 1 cm.
$+^1$ Indicates more than + and ± less than +.

perhaps double that amount. With the Dick test, the dosage can be standardized and the immunity results determined by retesting from time to time. The scarlet fever toxin is much less injurious to the local tissues than the diphtheria toxin, so that there is no need of adding antitoxin to it or of changing it by formaldehyd. Yet, as the Dicks state, such chemical modification, resulting in a decreased toxicity or toxoid, is desirable, if it can be demonstrated that it accomplishes immunization with fewer doses or in a shorter time. The most suitable dose of toxin varies with conditions. If scarlet fever is prevalent, it is very desirable not to give sufficient toxin to make a rash in any of the very susceptible children, for there is no certain way of determining in such a case whether it is a case of light scarlet fever or of a toxin rash. Even if cultures are made, it is possible that streptococci which produce the scarlet fever toxin will be present, and yet the case will be due to a toxic rash. If, however, no scarlet fever is in the neighborhood, a slight scarlet rash is of no importance and larger doses can be given.

TABLE 2.

RESULTS IN DICK POSITIVE CHILDREN THREE AND ONE-HALF MONTHS AFTER RECEIVING THREE IMMUNIZING INJECTIONS OF APPROXIMATELY 1,000, 2,000 AND 4,000 SKIN DOSES

	Reaction Three and One-Half Months Later	
11 $+ +$ or $+ +^1$	1 = +	63 per cent. immune
(Strongly positive)	3 = ±	27 per cent. doubtful
	7 = −	9 per cent. probably suspectible
10 $+$ or $+^1$	1 = ±	90 per cent. immune
(Moderate reactors)	*1 = pseudo	10 per cent. doubtful
	8 = −	
Total 21 cases	1 = +	76 per cent. immune
	4 = ±	19 per cent. doubtful
(Strong and moderate reactors)	15 = −	4 per cent. moderately susceptible
	1 ± pseudo negative	

* Gave + reaction with and without mixture with a sufficient quantity of convalescent serum to neutralize the toxin.

TABLE 3.

RESULTS OF FIVE IMMUNIZING INJECTIONS AT THE END OF THREE AND ONE-HALF MONTHS APPROXIMATELY 500, 1,000, 2,000, 3,000 AND 4,000 SKIN TEST DOSES

	After Three and One-Half Months	
32 $+ +$ or $+ +^1$	1 = +	81 per cent. immune
Strongly positive cases	5 = ±	= 17 per cent. probably immune
	23 = −	= 2 per cent. probably susceptible
	*3 + ± negative pseudo	
36 $+$ or $+^1$	34 = −	
Moderate reactors	*1 = + negative pseudo	100 per cent. immune
	*2 = ± negative pseudo	
69 $+$ to $+ +^1$	1 = +	91 per cent. immune
All positive reactors	5 = ±	7 per cent. probably immune
	57 = −	1 per cent. probably susceptible
	1 = + negative pseudo	
	5 = ± negative pseudo	

* 1 + and *5 ± are considered negative pseudo reactions because of an equal reaction in the control neutralized by convalescent serum.

Original Investigations.—Tables 1, 2, 3, 4 and 5 show the results in our tests following the injection of from 600 to 10,500 skin doses. The tests in these tables, with the exception of the one by Zingher, were made by Schroder and read by her, Williams and the Author.

The persistence of the acquired immunity following injections remain in doubt. With small injections, such as in the cases recorded in Table 5, many of the originally very positive cases have, after a year, relapsed, while the originally moderately positive cases have generally continued to hold their immunity or to increase it.

TABLE 4.

IMMUNITY RESULTS AFTER FOUR WEEKS WITH THREE INJECTIONS OF SCARLET FEVER TOXIN (DICK TEST)—1,200 SKIN TEST DOSES IN ALL (ZINGHER)*

Dick Retest After from Four to Five Weeks

			Dick Positive and Combined			Dick Negative and Pseudo	
Institution	Per Cent. Positive Dick at Original Test	Total Retested	Number Positive As in Original Test	Number Less Strongly Positive	Per Cent. Less Strongly Positive	Number	Per Cent.
Hebrew Orphan Asylum..	29.2	143	19	20	14.0	104	72.7
New York Orphanage....	44.4	91	10	36	39.5	45	49.4
Leak and Watts Home....	22.0	40	12	10	25.0	18	45.0
Total		274	41	66	24.0	167	61.0

* Doses given: under 12 years, 200, 500 and 500 for skin test doses at weekly intervals; over 12 years, 200, 500 and 1,000 skin test doses at weekly intervals.

TABLE 5.

RESULTS OF INJECTING 150, 300 AND 600 SKIN TEST DOSES AS SHOWN BY RETEST FIVE AND THIRTEEN MONTHS AFTERWARDS AT ST. DOMINICKS

	Five Months After	Per Ct.	Thirteen Months After	Per Ct.
One hundred positive cases	+ + = 0		+ + =	20
of St. Dominicks Test	+ = 8 =	9.8	+ =	9 susceptible
by Dr. Schroder	+ = 27 =	33.3	∓ =	14 doubtful
	± and — = 46 =	56.8	± or — =	57 immune
	Total 81		Total 100	

With much larger injections, such as those recorded in Tables 2 and 3, the immunity response is so much greater that it is hoped that the marked positive cases will not only develop immunity but hold it for a period of years.

We note in these tables that the moderately reacting cases develop complete immunity much more readily than the strongly reacting cases; again, that the response is quicker than after diphtheria toxin-antitoxin or toxoid injections. This corroborates the views of Gabritschewsky and the statements of the Dicks that immunity develops in many within one or two weeks.

The results of a second series of injections in those who have not responded to the first series have been excellent.

The persistence of a negative Dick reaction in those found to be negative: Observations on several thousand children by Zingher and Schroder at different intervals of time up to thirteen months have shown that a naturally acquired immunity is usually persistent for at least one year. Only about 2 per cent. of those originally negative have been found to change. It is fair to assume, therefore, that as in the case of naturally acquired immunity to diphtheria, the immunity to scarlet fever naturally acquired is apt to remain. This finding, which is in accord with the findings of the Dicks, encourages us to hope that, with larger and perhaps more numerous doses, even the very positive cases may acquire such a high degree of immunity that it will last for at least a few years. We already have the knowledge that these immunizing injections are very useful in stopping an outbreak of scarlet fever.

The Serum Treatment of Scarlet Fever as Observed at the Willard Parker Hospital

Scarlet fever antitoxin was first administered by us in January, 1924. This was made possible through a supply of serum by Dr. Dochez. The first serum was only moderately potent and, in the doses given by the intramuscular method, the results though good were not striking. In January, 1925, it was decided to give it to alternate patients, and in severe cases to give it intravenously. The antitoxin most used was our own preparation obtained from two horses that had received increasing toxin injections for nine months and more; but in about one-fourth of the cases we used a new preparation of the serum sent us by Dochez. The result of the intravenous injection was so striking that the medical board decided in March, 1925, to give antitoxin to all patients having a rash and a temperature of 101 F. or higher and to give it to alternate patients having a temperature lower than 101. It was given intravenously in all severe cases.

The antitoxin serum was given by Dr. Mildred McBride, the assistant resident physician who was in charge of the scarlet fever service at the Willard Parker Hospital. The attending physician on duty and the Author watched with her the effects of the treatment. The potency of the antitoxic serum was tested either by the Schultz-Charlton blanching test or by the more accurate Dick neutralization tests of the toxin. The potency ranged from 100 to 300 units per cubic centimeter; that is, 1 cc. of the serum neutralized between 5,000 and 15,000 Dick test skin doses of toxin.

Most of the serum was not refined. About fifty patients, however, received the refined antitoxic globulin solution. The therapeutic results were alike, but rashes occurred in about 60 per cent. after the injection of the unrefined serum, and in only about 30 per cent. after injection of the refined. The rashes and other symptoms of serum disease that did occur were more severe with the unrefined. No serious after-effects occurred in any case, but the serum sickness in some was very annoying. The dosage was determined by much the same principles that govern the dosage of diphtheria antitoxin. A sufficient dose should be given at the earliest possible moment. An amount should be given in the first dose to make and keep the fluids of the body antitoxic. If, however, the temperature rises after the drop, a second injection should be given. The size of the dose is influenced by the weight of the individual and the severity of the case. Whether a late case in which there is still a marked rash should be treated with larger doses than an early case of equal severity is in our belief doubtful and remains still a matter for further investigation. Intravenous injections of sufficient size give the most striking results. The fluids of the body become quickly antitoxic, as shown by the Dick test. This, when done even an hour or two afterward, is always negative, if a sufficient dose has been given, and remains negative if done later in the disease.

The results from sufficiently large intramuscular injections are certain, but they develop more slowly. The Dick test is positive for from six to twelve hours after an intramuscular injection. It then becomes negative.

The results as noted at the bedside in the majority of patients are very striking. The higher the temperature and the more toxic the case, the more striking will be the results, if the serum is given very early in the disease. After an intravenous injection in an early uncomplicated case, the patient, as a rule, finds within a few hours that the throat is less sore, the mind clears, vomiting ceases, the appetite returns, and the temperature and pulse begin to fall. Within from six to eight hours, a delirious and a very sick patient is often convalescent. With a larger dose given intramuscularly the same results follow, but more slowly. With insufficient dosage there is a less rapid improvement, and the toxic symptoms may return. Our results were practically identical with those reported by Blake and Birkhaug.

The following two cases showed favorable results after intramuscular injections in the first and intravenous injection in the second:

Case 1.—G. C., a boy aged 7, was sick two days with headache and vomiting. A rash developed on the morning of the 23rd. On admission in the afternoon, the temperature was 103 F.; pulse rate, 150; rash marked, and throat inflamed. The Dick test made on admission was positive. The severity was from moderate to severe. The patient was given intramuscularly 40 cc. (about 3,200 units) of Dochez' antiscarlatinal serum. On the 24th the rash was greatly lessened; the Dick test made in the morning was negative, and the temperature was 101 F. in the morning and 100 F. in the afternoon. On the 25th the rash was gone; the temperature, 99.6 F. The patient made an eventful recovery (Chart 1).

Case 2.—A. K., a man aged 32, who had a sore throat on the 17th, with a rash appearing on the 19th, was admitted early in the afternoon on the 20th. The temperature was 102 F. and rose to 104 F.; the pulse rate was 130. The throat was inflamed. Adenitis was present, and the patient was fairly toxic. He received, on admission, 2,400 units of antitoxic serum intravenously. Within two hours he felt improvement. At the end of six hours the temperature had fallen to 98.8 F., the rash had faded and the patient felt almost well. Convalescence was uncomplicated (Chart 1).

Chart 1 is characteristic of uncomplicated early cases of scarlet fever that respond to the serum treatment. In many there is no subsequent rise of temperature. In others, because of complications or serum sickness, the convalescence is interrupted.

Chart 2 gives the temperatures of five patients who received intramuscular injections. It will be noted, as in Case 1 the previous chart, that the drop in temperature is comparatively slow. Two of these patients received insufficient amounts of antitoxin.

Chart 3 gives the temperatures in five early cases of scarlet fever in which intravenous injections were given and the patients reacted favorably. The temperatures of an untreated case are given also for comparison. The rapid drop of the temperature in four of the patients is striking. In the fifth case only 1,500 units was given. The rash remained bright, and the temperature rose again.

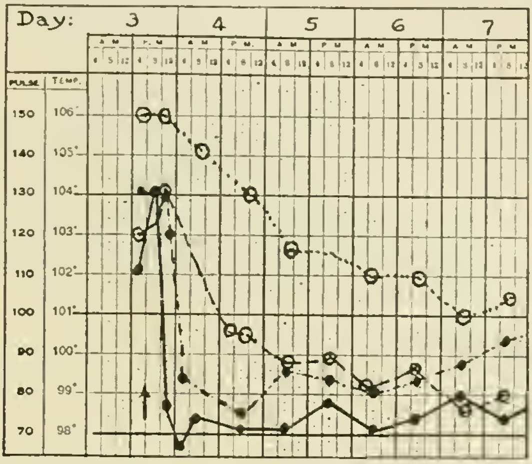

Chart 1.—Pulse and temperature changes in Cases 1 and 2; solid line with solid dots, temperature curve, intravenous injection; broken line, hollow dots, temperature curve, intramuscular injection; broken line, solid dots, pulse curve, intravenous injection; dotted line, hollow dots, pulse curve, intramuscular injection; arrow indicates serum injection.

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Chart 4 gives the composite temperature curves of twenty-five severe, twenty-five moderate and twenty-five light cases of scarlet fever treated with serum during the first three days of the disease and an equal number without.

Complications.—The antitoxin has little effect on complications, such as infection in the tonsils, in the middle ear, in the mastoid, in glands of the

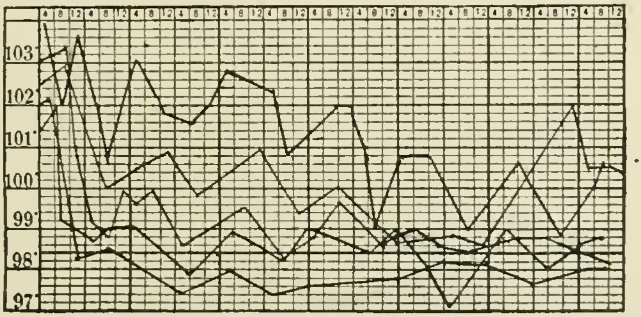

Chart 2.—Temperature curves in five uncomplicated cases after an intramuscular injection of antitoxin. (Reprinted by courtesy of The Journal of The American Medical Association.)

neck or in the endocardium, when they have already developed; but before they have developed the antitoxin appears to be of service in preventing their occurrence. This is probably due to the raising of the resistance of the local tissues and of the body as a whole so as frequently to prevent or restrict the invasion of the body by the streptococci. These streptococci may be those producing the scarlet fever or other varieties. An interesting case was that of a woman sick with toxic scarlet fever complicated by severe erysipelas. The delirium vanished, the vomiting ceased, the temperature was lowered and the rash faded, but the erysipelas ran its usual course. Table 6, which shows the complications developing in seventy-five cases that were treated and in seventy-five that were untreated, indicates a favorable influence of the antitoxin in preventing complications. As long as the rash remains, antitoxin is indicated.

TABLE 6.

COMPLICATIONS DEVELOPING IN SCARLET FEVER IN SEVENTY-FIVE CASES UNTREATED WITH SERUM AND IN SEVENTY-FIVE EARLY CASES TREATED WITH SERUM

	Nephritis	Otitis	Adentis	Arthritis	Mastoiditis
Twenty-Five Severe Cases:					
Without serum	9	12	8	9	5
With serum	2	6	11	5	3
Twenty-Five Moderate Cases:					
Without serum	9	8	8	8	0
With serum	0	7	11	4	0
Twenty-Five Mild Cases:					
Without serum	4	0	8	3	0
With serum	0	4	6	2	0

The cases in Table 6 are not strictly comparable, since the severe and moderate cases which were not treated with serum are taken from 1924, while the serum-treated cases occurred in 1925. The diagnosis of nephritis is based mostly on the urine examinations and is probably not wholly accurate. The kidney complications, on the whole, are seen to be decidedly less in the cases treated with antitoxin early, but the other complications are about the same.

Reduction in Mortality.—During the year 1924, when scarlet fever was very light, 569 patients with scarlet fever were admitted to the hospital. About forty of these received scarlet fever antitoxin. The mortality among the 569 patients was 2.8 per cent. During April and May of this year, when all patients with cases of any severity and with a rash were receiving serum, there were 220 admissions. Of these, six patients, or a percentage of 2.7, died.

These figures, considered without a knowledge of the cases, would be very misleading; but they cause one to wonder what proportion of the 150 or more deaths from scarlet fever which occur yearly in New York can be eliminated by the use of serum. In three of the six fatal cases the patients were admitted so late that they were absolutely unsuitable for antitoxin, but in three of them they were admitted on the third day. The histories of these six fatal cases are as follows:

Case 3.—A child aged 11, admitted thirty-eight days after the development of scarlet fever, showed symptoms of meningitis. Death occurred three weeks later. Necropsy showed several small brain abscesses.

Case 4.—A child, aged 7, admitted on the ninth day of disease, with double otitis media, septicemia and signs of endocarditis, died in three days.

Case 5.—A man, aged 35, admitted in the afternoon on the sixth day of the disease, with an intense rash still present, and a temperature of 104.8 F., received 60 c.c. of Dochez serum intravenously (about 5,000 units). The temperature dropped within six hours to 99.5 but rose the following afternoon when he died. Necropsy showed purulent bronchitis and toxic changes.

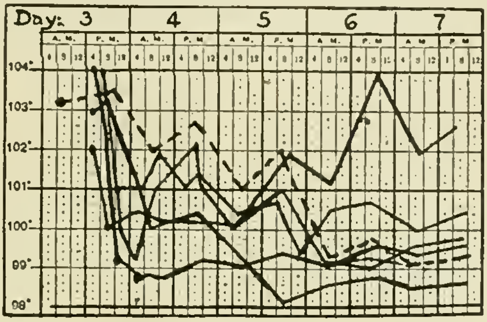

Chart 3.—Temperature curves in five early cases after an intravenous injection of antitoxin, and in one untreated case. The continuous lines give the temperature curves of the patients treated by intravenous injections of antitoxin; one patient received too small an injection (1,500 units); the broken line is the temperature curve of an untreated patient.

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Case 6.—A patient, aged 23, ill three days, with a marked rash, marked pharyngitis with grayish exudate and a temperature of 103.6 F., received 30 c.c. of serum intravenously (about 1,800 units). Complete anuria was present during the first thirty-six hours. The temperature dropped to 99 in eight hours following the administration of the serum, but rose again. The rash remained pronounced for three days. The patient died on the fourth day after admission.

Case 7.—A patient, aged 21, following a kidney operation at another hospital, developed scarlet fever, and was sick three days before admission. The patient appeared toxic with a generalized hemorrhagic rash. The temperature was 103 F. The patient was given 30 c.c. of serum (about 2,000 units) intravenously. The temperature dropped 4 degrees in eight hours, but rose

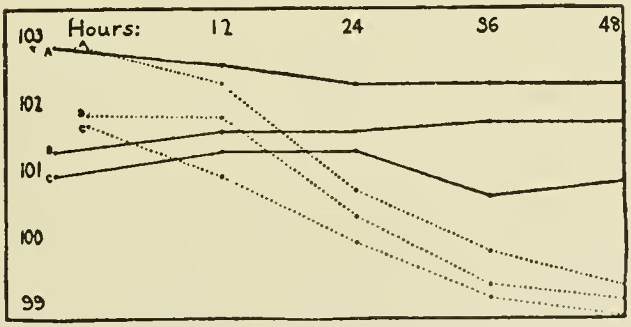

Chart 4.—Average temperatures of seventy-five patients treated with serum and seventy-five patients treated without serum; solid line, patients treated without serum; dotted line, patients treated with serum. There were twenty-five cases of each: *A*, severe; *B*, moderate; *C*, mild.

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in the afternoon just before death. The rash remained, but was somewhat faded.

Case 8.—J. M., aged 3½ years, was taken ill with vomiting, May 10, developed a rash on the 12th, and was admitted on the 13th, with a temperature of 104.8 F., a generalized hemorrhagic rash and an intense pharyngitis with exudate on the tonsils. Several hours after admission, 30 c.c. of antitoxic serum (about 2,400 units) was given intravenously. The temperature dropped to 100 F., but rose to 102 in afternoon. Another 40 c.c. of serum was given intravenously. The temperature dropped to 101, but rose in the evening to 103 and continued between 101 and 103 until death, May 20. The rash did not fade until the 19th. Necropsy revealed an ulcerative inflammation of the mouth, esophagus and stomach.

In the last three of the six fatal cases, the serum was given about as quickly as would generally be possible. Nevertheless, the patients responded only temporarily and inadequately to the antitoxic serum. It is possible that if the serum had been given immediately after the development of the rash, a better result might have been obtained or perhaps much larger or repeated doses of antitoxin might have done more good. There is also the possibility that some of the cases presenting septic rashes are not ordinary scarlet fever and are due to streptococci that differ in the type of their toxin from those produced by the ordinary strains. We have evidence of such difference, but unfortunately we did not isolate and test the streptococci from these cases for their toxins.

Conclusions on the Therapeutic Value of Antitoxin

The antiscarlatinal serum produced by Moser and Savchenko produced the same therapeutic results as that now produced by the Dochez method or by the subcutaneous injection of known doses of toxin. The value of the earlier products was greatly handicapped by the lack of any means such as the Schultz-Charlton and Dick methods for estimating their antitoxic potency. Because of this lack of knowledge, serum weak in antitoxin was undoubtedly frequently used with disappointing results.

The antitoxic serum should be given in sufficient amount as early in the disease as possible. When thus given, the results in most cases are strikingly favorable. The early use of antitoxin probably frequently prevents the development of complications.

Antitoxin is useless after the rash has disappeared and has no effect on the later septic complications.

In moderate cases it should be given intramuscularly. In severe or toxic cases it should be given intravenously. As a rule, a single dose of sufficient size is enough, but in severe cases in which the symptoms return, a second dose in from twelve to twenty-four hours is often desirable. It is too early to state whether the serum should be given in very mild cases, since the serum sickness is often more annoying than the scarlet fever.

The refined antitoxic serum produces fewer and less severe rashes than the unrefined, and should in time be the only preparation used. A unit should be adopted, so that results from different dosages may be compared. The one suggested at a recent meeting of immunologists in Washington seems to be suitable. One unit is defined as the amount of antitoxin that neutralizes 50 skin test doses (Dick), as determined by an intracutaneous test in man. A dosage between 2,000 and 10,000 units will probably suffice in the great majority of cases. A few of the failures of antitoxin may be due to the fact that some toxic cases may be due to streptococci that produce other toxins than are neutralized by the antitoxin used. This is an interesting problem for further study. Whether it will be of marked advantage to combine an antimicrobial serum with the antitoxic serum is still doubtful, but merits careful investigation.

Such a serum used alone in the past has given doubtful results, but combined with antitoxin it may add to the benefit produced. There would thus be a return to a serum such as was used by Moser, but with knowledge of the potency in both the bactericidal and the antitoxic properties.

For bibliography see the original article.

THE DICK TEST IN NORMAL PERSONS AND IN ACUTE AND CONVALESCENT CASES OF SCARLET FEVER

Immunity Results With Scarlet Fever Toxin

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Abraham Zingher

Results with the Dick Test in Normal Persons

The test has been applied to the following groups of individuals, and their reactions have been studied according to age and social conditions:

1. Mothers and young infants.
2. Children of nursery and preschool age.
3. Elementary and high school children.
4. Adults.
5. Certain selected groups: children in private schools, nurses in training schools and individual families.

1. *Mothers and Young Infants.*—This study was undertaken to determine whether there was an intra-uterine transmission of scarlatinal antitoxic antibodies similar to the known transmission of diphtheria antitoxin from mother to offspring through the placental circulation.

Table 1 shows the similarity in the scarlatinal antitoxic immunity of mother and infant. The infant usually retains its immunity up to the fifth or sixth month of life, and then begins to lose it. Occasionally, an infant is seen who does not lose the antitoxic immunity until he approaches the end of the first year.

The positive reactions in young infants are much smaller and less intense than similar reactions in older children. We have noted the same phenomenon with the Schick test in young infants. The slight reactions are probably due to the limited ability of the skin of young infants to respond to inflammatory stimuli. It is due either to the slow development of this mechanism in the skin, which appears to some extent only after the infants have become a few months old, or to a natural defense mechanism with which Nature endows the young infants to protect them at the most tender period of their lives. The first explanation is probably the more correct one.

In two groups of new-born infants the Author examined specimens of blood obtained from the umbilical cord of the placenta. In one group, five of the six specimens showed the presence of antitoxin. In another group, four out of six specimens also had antitoxin. In this group the mothers and infants had also received the Dick test. In each instance in which antitoxin was present in the placental blood, the mother and infant gave a negative reaction. In the two cases in which no antitoxin could be detected, both mother and infant gave positive Dick reactions. Scarlet fever is thus the second disease, the first one being diphtheria, in which a definite placental transmission of antitoxic antibodies has been demonstrated. This is important additional evidence that we are probably dealing with similar placental transmission of antibodies against other infectious diseases, such as measles and poliomyelitis.

TABLE 1.

THE DICK TEST IN MOTHERS AND INFANTS SHOWING MATERNAL TRANSMISSION OF SCARLATINAL ANTITOXIC ANTIBODIES

0-1 Month	1-2 Months	2-3 Months	3-4 Months	4-5 Months	5-6 Months	6-7 Months
{14 days .... — Mother ... —	{5 weeks .... — Mother .... —	{2 months ... + Mother .... +	2{3.5 months .. — Mother .... —	{5 months ... — Mother .... —	{5.5 months .. $\underline{\pm}$ Mother .... —	{7 months ... $\underline{\pm}$ Mother .... —
{23 days .... — Mother .... —	{5 weeks .... + Mother .... —	{2.5 months .. — Mother .... —	3{4 weeks .... — Mother .... —	2{5 months ... ± Mother .... —	{6 months ... + Mother .... —	
{26 days .... — Mother .... —	{6 weeks .... — Mother .... —					
{28 days .... — Mother .... —	2{7 weeks .... + Mother .... +					

7-8 Months	8-9 Months	9-10 Months	10-11 Months	11-12 Months	12-18 Months	18-24 Months
{7.5 months .. $\underline{\pm}$ Mother .... —	{9 months ... — Mother .... —	{10 months .. ± Mother .... —	{11 months .. + Mother .... +	{12 months .. $\underline{\pm}$ Mother .... —	{13 months .. + Mother .... —	{19 months .. + Mother .... —
*2{8 months ... + Mother .... —						{22 months .. + Mother .... +

* Indicates two similar groups observed; +, ± and $\underline{\pm}$ indicate varying degrees of positive reaction; — indicates a negative reaction.

2. *Age Groups.*—More than 4,500 persons who received the Dick test are classified by age groups in Table 2. These tests were made on children and adults in various institutions and in two public schools.

It is interesting to note in Table 2 the susceptibility to scarlet fever of individuals of different age groups. This closely resembles the susceptibility to diphtheria as determined with the Schick test. Other factors, however, besides age, play a significant part in determining the susceptibility of different persons to scarlet fever. These factors we have also found to be important in connection with the susceptibility to diphtheria.

TABLE 2.

THE DICK TEST AT DIFFERENT AGE GROUPS

Age	Total Tested	Dick Positive	Dick Negative	Per Cent. Dick Positive
0- 6 months	29	13	16	44.8
6-12 months	42	27	15	64.2
1- 2 years	123	87	36	70.7
2- 3 years	140	95	45	67.8
3- 4 years	207	123	84	59.4
4- 5 years	237	110	127	46.4
5-10 years	1,475	522	953	35.4
10-15 years	1,690	430	1,260	25.4
15-20 years	285	75	210	26.3
20 years up	342	61	281	17.9
Total	4,570	1,543	3,027	34.4

Table 3 shows the very high degree of susceptibility to scarlet fever among children of the more well-to-do classes in our population.

3. *Social Groups.*—Table 3 shows the results with the Dick test in two private schools. The average of positive Dick reactions among these 320 children was over 83 per cent. Even in the group between 11 and 15 years of age, the positive reactors were more than 81 per cent. This explains the high incidence of scarlet fever, once it breaks out in a school of this kind. At the Riverside Country School there were, during the past winter

TABLE 3.

THE DICK TEST IN TWO PRIVATE SCHOOLS (RIVERDALE COUNTRY SCHOOL AND HORACE MANN SCHOOL, COLUMBIA UNIVERSITY)

Age	Total Tested	Dick Positive	Negative Dick	Per Cent. Dick Positive
4 years	9	9	0	100.0
5 years	16	14	2	87.5
6 years	15	12	3	80.0
7 years	25	21	4	84.0
8 years	32	28	4	87.5
9 years	19	15	4	78.9
10 years	38	37	1	97.3
11-15 years	119	97	22	81.5
16-20 years	47	35	12	74.4
Total	320	268	52	83.7

and spring, twenty-three cases of scarlet fever out of a total school population of 125 boys varying in age between 10 and 17 years. Of seventy-one boys tested in this school, fifty-one gave a positive Dick reaction. Of the twenty negative reactors, six had scarlet fever, three during the recent epidemic and three in childhood. The Author found a similar high degree of susceptibility to diphtheria among the children of the more well-to-do of New York City.

Table 4 shows by contrast the susceptibility of the children in a public school in the borough of the Bronx. Only 22.07 per cent. gave a positive Dick reaction. It is interesting to note that in this school we had found 21.6 per cent. of the children positive to the Schick test.

TABLE 4.

RESULTS WITH THE DICK TEST AT PUBLIC SCHOOL NO. 4, BRONX

	Nonimmunes			Immunes			Total Tested	
Age	Positive	Combined	Total	Negative	Negative-pseudo	Total	Number Tested	Per Cent. Positive
5- 6	14	1	15	6	3	9	24	62.50
6- 7	18	1	19	15	3	18	37	51.35
7- 8	11	3	14	18	10	28	42	33.33
8- 9	16	8	24	24	20	44	68	35.29
9-10	6	6	12	31	17	48	60	20.00
10-15	20	26	46	175	137	312	358	12.85
Total	85	45	130	269	190	459	589	22.07

Schick Test for Diphtheria Susceptibility or Immunity at Public School No. 4, Bronx

Total	Shick Positive	Per Cent. Schick Positive
1,133	248	21.6

Table 5 shows the percentage of positive Dick reactors among children in various institutions that I tested in New York City, Rochester and Buffalo.

Table 6 gives the percentage of positive Dick reactors among nurses in different hospitals and among the students at Teachers College, Columbia University. It is interesting and rather significant to note the high proportion of susceptible persons in these groups of adults. This is accounted for by the fact that many of the nurses come into training schools from small communities where there has been comparatively little exposure to infection with the scarlet fever hemolytic streptococcus. The Lenox Hill Hospital, with many of its pupils from larger cities, shows the smallest percentage of positive Dick reactors. The percentage of positive Schick reactors in each of these institutions is also very high.

TABLE 5.

THE DICK REACTION IN DIFFERENT INSTITUTIONS

Institutions	Age in Years	Nonimmune			Immune			Total Tested	
		Dick Positive	Combined	Total Positive	Dick Negative	Pseudo	Total Negative	Total Tested	Per-Cent. Positive
Heckscher Foundation	2 wk.–5 yr.	39	2	41	31	1	32	73	56.1
Jewish Orphan Home, Rochester, N. Y.	8–16	10	7	17	6	15	21	38	44.6
Hillside House, Rochester, N. Y.	3–16	63	11	74	60	32	92	166	44.5
N. Y. Orphanage, Hastings, N. Y.	3–16	59	29	88	91	19	110	198	44.4
Society Prevention of Cruelty to Children, N. Y.	3–15	31	11	42	58	34	92	134	31.3
St. Mary's Orphan Asylum, Rochester, N. Y.	4–15	32	18	50	64	47	111	161	31.0
Hebrew Orphan Asylum, N. Y.	3–17	131	67	198	309	171	480	678	29.2
Leak and Watts Orphan Home, Yonkers, N. Y.	3–17	86	45	130	269	190	459	589	22.0
German Roman Catholic Orphan Home, Buffalo, N. Y	2–16	81	8	89	270	57	327	416	21.1
Total		531	198	729	1,158	666	1,724	2,453	29.3

TABLE 6.

THE DICK REACTION ON NURSES AT DIFFERENT HOSPITALS AND STUDENTS AT TEACHERS COLLEGE

Institutions	Nonimmunes			Immunes			Total Tested		Result with Schick Test Per Cent. Schick Positive
	Dick Positive	Positive Combined	Total Positive	Dick Negative	Negative pseudo	Total Negative	Total Tested	Per Cent Dick Positive	
Students at Teachers College, Columbia Univ.	21	9	30	20	2	22	41	73.1	65.0
General Hospital, Rochester, N. Y.	7	8	15	10	2	12	27	55.5	75.0
St. Vincent's Hospital	37	5	42	28	10	38	80	62.5	55.0
Children's Hospital, Buffalo, N. Y.	7	4	11	11	1	12	23	47.8	60.0
New York Hospital	20	7	27	23	16	38	65	41.5	54.3
Lenox Hill Hospital	16	19	35	42	15	67	92	38.0	43.7
Total	108	62	160	134	45	179	328	48.7	

4. *In Family Groups.*—Table 7 gives the results with the Dick test in the families of six physicians. Some of the children and adults had a negative reaction with no history of scarlet fever. In the first family, that of Dr. T. E. W., the boy of 11 years had had scarlet fever recently, and on his return home gave the disease to the 10-year-old boy. The Dick test showed

TABLE 7.

THE DICK REACTION IN FAMILIES

Families	Age	Dick Positive	Dick Negative	Comment
1. Dr. T. E. W.	..	..	—	No history of scarlet fever
Mrs. T. E. W.	..	..	—	No history of scarlet fever
Tom	12	..	—	No history of scarlet fever
Frank	11	..	—	Scarlet fever, 44 days convalescence
Donald	10	..	—	Scarlet fever, 30 days convalescence
George	8	+	..	
Jack	6	+	..	
Kenneth	5	+	..	
Arthur	2	+	..	
2. Dr. J. B.	..	..	—	No history of scarlet fever
Mrs. J. B.	..	..	—	No history of scarlet fever
Margaret	15	±	..	
James	13	++	..	
Elizabeth	10	+	..	
Jeanne	7	..	—	No history of scarlet fever
Anne	18 m	+	..	
3. Dr. R. F.	..	..	—	No history of scarlet fever
Mrs. R. F.	..	+	..	
John	8	+	..	
Mary	5	+	..	
William	2	+	..	
4. Dr. H. E.	..	..	—	History of scarlet fever
Mrs. H. E.	..	+	..	
Ethel	22	+	..	
Robert	20	..	—	No history of scarlet fever
Haven	18	+	..	
Ruth	13	+	..	
Ralph	12	+	..	
5. Dr. S. W.	..	..	—	History of scarlet fever
Mrs. S. W.	..	..	—	No history of scarlet fever
Shirley	14	..	—	History of scarlet fever
Ethel	12	..	—	History of scarlet fever
Harmon	20 m	+	..	
6. Dr. A. Z.	..	..	—	No history of scarlet fever
Mrs. A. Z.	.	..	—	No history of scarlet fever
Joseph	4	+	..	
Diana	2	+	..	
Henry	11 m	+	..	

that the parents and the oldest boy were immune. All three gave a negative history of scarlet fever. The two convalescent boys also gave a negative reaction, while the four younger boys gave a positive reaction. The positive Dick reactors in all these families have been injected with increasing doses of scarlet fever toxin for the purpose of active immunization. Group 6 represents the Author's own family. The three children were also actively immunized with diphtheria toxin-anti-toxin at the age of 8 months. All three give a negative Schick reaction at the present time.

5. *Comparison Between Dick Test and Schick Test in the Same Person.*—Table 8 shows various groups of persons who were tested with both the Dick test and the Schick test. It is interesting to note that about two-thirds of the total number had similar reactions. At Public School 4, Bronx, the large majority was negative to both tests; in the private school and among the nurses at the hospitals, a very large proportion was positive to both tests. When the two reactions were different, there was approximately the same number in each group, with a slightly larger proportion in a group of negative Dick and positive Schick reactors. The repeated exposure to contact infection, with resultant development of natural immunity, need not, of course, have been equal in degree and frequency to both diphtheria and scarlet fever.

TABLE 8.

COMPARISON BETWEEN DICK TEST AND SCHICK TEST IN THE SAME PERSONS

School or Institution	Total Tested	Reactions Similar				Reactions Not Similar			
		Dick Positive Schick Positive		Dick Negative Schick Negative		Dick Positive Schick Negative		Dick Negative Schick Positive	
		Number Tested	Per Cent. Total Tested	Number Tested	Per Cent. Total Tested	Number Tested	Per Cent. Total Tested	Number Tested	Per Cent. Total Tested
Horace Mann School, Columbia College	144	107	74.3	7	4.8	15	10.4	15	10.4
Lenox Hill Hospital, nurses	23	11	47.8	0	0	2	8.6	10	43.4
General Hospital, Rochester, N. Y., nurses	23	11	47.0	0	0	2	8.6	10	43.4
Children's Hospital, Buffalo, N. Y., nurses	23	11	35.0	5	25.0	3	15.0	5	25.0
Teachers College, Columbia University	29	10	34.4	7	24.1	4	13.7	8	27.5
New York Hospital, nurses	38	10	26.3	9	23.6	9	23.6	10	26.3
St. Vincent's Hospital, nurses	71	16	22.5	22	30.9	23	32.3	10	14.4
Public School 4, Bronx	219	13	5.9	147	67.1	24	10.9	35	15.9
Total	567	185		197		82		103	
Per Cent. of total tested			32.6		34.7		14.4		18.1

Results With the Dick Test in Acute and Convalescent Cases of Scarlet Fever

During the last three months we have given the Dick test to all scarlet fever patients admitted to the Willard Parker Hospital. Most of the patients were tested on admission, and subsequently at intervals of several days. The results have been very striking, and afford good evidence of the value and reliability of the Dick test as an index of susceptibility or immunity to scarlet fever.

These observations are shown in Tables 9, 10 and 11.

1. *Summary of Results in Scarlet Fever.*—Table 9 gives a summary of the results with the Dick test in acute and convalescent cases of scarlet fever. In the first part of the table, we note that every one of the 141 patients tested during the first five days of illness had a positive reaction. The degree of reaction varied, most of the patients giving a + or ± reaction. The presence of a + + reaction after the third day of scarlet fever should raise strong doubts whether we are dealing with true scarlet fever, or at least with the usual scarlet fever virus. This doubt would be more than justified if the reaction continues strongly positive into convalescence. Among forty-nine patients admitted with a history of six days or more illness, three gave a ± and five a ⩲ reaction. The history of the onset of the disease in many of the patients is not always reliable.

TABLE 9.

SUMMARY OR RESULTS WITH DICK TEST IN ACUTE AND CONVALESCENT CASES OF SCARLET FEVER

1. Results of First Dick Test on Scarlet Fever Patients at Time of Admission to Hospital.

Number Days of Illness Before Dick Test	Reactions Tested					Total Tested		
	++	+	±	⩲	—	Number	Dick Positive	Per Cent. Dick Positive
1-5	8	62	66	5	0	141	141	100.0
6 and over	0	0	3	5	41	49	8	16.5
Total	8	62	69	10	41	190	149	78.4

2. Total Number of Patients Receiving Dick Test During Acute Stage and Convalescence from Scarlet Fever

A.	Total number tested	170
B.	Total number Dick positive on admission to hospital and Dick negative during convalescence	158
C.	Total number Dick positive on admission to hospital and Dick positive during convalescence	12
D.	Per cent. giving negative Dick reaction during convalescence from scarlet fever	93.0

In the second part of the table are enumerated the patients who were tested on admission to the hospital and later during convalescence. Of the 170 tested, 158, or 93 per cent., gave a positive reaction earlier in the disease and a negative reaction during convalescence. There were, in addition, twelve patients who had a positive Dick reaction during convalescence. By using a

TABLE 10.

THE DICK TEST IN SCARLET FEVER PATIENTS DURING ACUTE AND CONVALESCENT STAGES OF THE DISEASE. PATIENTS SHOWING POSITIVE DICK REACTIONS DURING CONVALESCENCE FROM SCARLET FEVER

Name	Age	Date of Admission	No. of Days Ill Before Admission	Dick Tests					Comment
				First	Second	Third	Fourth	Fifth	
Mary F.	40	4/5	2	4/5+	4/10+	4/25+	......	......	On admission, Schultz-Charlton test with convalescent and Dochez serum moderately positive
Catherine S. ..	26	4/6	2	4/6±	4/10±	4/25±	......	......	On admission, Schultz-Charlton test with convalescent and Dochez serum moderately positive
Paula M.	5	3/16	2	3/17±	3/31—	4/10±	4/25+	......	Desquamation present
Dorothy F. ..	4	3/28	4	3/28+	3/31—	4/10±	4/25+	......	Slight desquamation on one hand; none on feet
Nema T.	28	5/17	2	5/17++	5/20+	5/22+	5/23+	5/28+	Nonscarlatinal patchy desquamation
Ray H.	7.5	4/11	2	4/13+	4/25±	4/30—	5/3±	5/10+	No desquamation. Blood taken 5/11 did not neutralize scarlet fever toxin and did not blanch rash
Tessie L.	4.5	4/6	3	4/6++	4/10++	4/25++	5/3++	5/10++	No desquamation. Blood taken 5/11 did not neutralize scarlet fever toxin and did not blanch rash
Rebecca K. ...	20	4/15	4	4/16+	4/19±	4/25±	5/3±	......	No desquamation
Margaret B. ..	2	2/15	2	......	3/6—±	3/11±	......	......	No desquamation
Isidore B.	23	3/10	2	3/11±	3/22±	4/1±	......	......	No desquamation
Mary B.	4	4/19	4	4/19+	4/25+	5/3±	5/10±	......	No desquamation
Margaret H. ..	20	4/4	5	4/4±	4/15±	4/25+	......	......	No desquamation

TABLE 11.

PATIENTS DEVELOPING SCARLET FEVER AFTER ADMISSION TO HOSPITAL

Name	Age, Years	Date of Admission	Number of Days Ill Before Admission	Dick Test on Admission		Attack of Scarlet Fever	Dick Retests During Convalescence				
				First	Second		Third	Fourth	Fifth	Sixth	Sevent
Verna B.	10	4/23	10	4/24++	4/25++	4/28	5/3+	5/4—	5/8— Ps.	5/10—Ps.	5/15—
Raymond W.	1.5	5/13	4	5/13++	5/17++	5/18	5/20+	5/22—	5/24—	5/31—	

stronger toxin dilution, it is possible, of course, to test convalescent patients, and obtain positive reactions in persons who have given a negative reaction with the standard toxin dilution. In fact, the antitoxin content of the blood of convalescents can be directly estimated in this way. The important question that has to be considered, however, is the significance of the persistent positive Dick reaction obtained with a standard toxin dilution in patients convalescent from scarlet fever.

2. *Group of Irregular Cases.*—Table 10 shows the twelve persistent positive Dick reactors. Of the twelve, the last seven enumerated had no desquamation during convalescence. Of the first four, Patient 1 and Patient 2 had shown a positive blanching test with convalescent serum and Dochez horse serum. Patient 3 had moderate desquamation, and Patient 4 slight desquamation. Patient 5 showed a patchy desquamation over both shoulders and arms, but no desquamation on the palms of the hands or the soles of the feet. Blood taken on the thirteenth day of illness, May 26, 1924, was negative for antitoxic antibodies. The interesting question has to be considered whether desquamation is a sine qua non in the diagnosis of scarlet fever.

3. *Group of Patients Developing Scarlet Fever After Admission to the Hospital.*—Table 11 shows in a very striking way the diagnostic value of the Dick test. The two patients were admitted to the Willard Parker Hospital with the diagnosis of scarlet fever. Neither one of these patients had the disease on admission, for they both developed scarlet fever five days after entering the hospital. The first patient (Verna B.) came in with a history of scarlet fever of ten days' duration. The Dick reaction on the second and the third days after admission was strongly positive (++). Such a strongly positive reaction on the twelfth and the thirteenth days of illness led the Author to make a diagnosis of "no scarlet fever." Two days later the patient developed a typical attack of the disease. The Dick reactions, made five and six days after the appearance of the rash, were slightly positive. On the tenth day the Dick reaction was negative and continued negative throughout convalescence.

The second patient (Raymond W.) also showed a strongly positive Dick test on the second and fourth days after admission to the hospital. These days would correspond to the sixth and eight days of the supposed illness, a time when the Dick reaction is usually slightly positive or negative in a true case of scarlet fever. On the fifth day after admission to the hospital the boy developed a typical scarlet fever rash. Two days later the Dick reaction was still positive. However, four days later, the reaction was only slightly positive and six days later it was negative.

A strongly positive Dick reaction noted in a case of scarlet fever after the patient has had the disease for a few days should put one on guard as to the correctness of the diagnosis. According to Pirquet, after the period of incubation of an infectious disease, during which the organisms multiply in the body and stimulate the development and gradual accumulation of antibodies, the period of invasion is ushered in by the interaction between the organisms and the antibodies that have been gradually produced. Antitoxic antibodies, present in small amounts during the early stages of scarlet fever, are sufficient to prevent the development of strong positive Dick reactions (Table 12) similar to the ones seen in normal, susceptible persons; only moderately or slightly positive reactions are noted. The Dick reaction soon becomes negative as the disease progresses into convalescence.

TABLE 12.
THE DICK REACTION IN SCARLET FEVER AND ITS RELATION TO THE SCHULTZ-CHARLTON PHENOMENON

A. The Dick Reaction in Normal Susceptible Persons and in Scarlet Fever Cases

	Scarlet Fever			
Normal Person	Period of Incubation	Period of Invasion	Period of Eruption	Period of Convalescence
++	++ changing to +	+ changing to ±	± changing to $\underline{\pm}$	$\underline{\pm}$ changing to –

++, strongly positive; +, positive; ±, slightly positive; $\underline{\pm}$, very faintly positive; –, negative.

B. The Dick Test and Schultz-Charlton Phenomenon in Scarlet Fever

	Days of Illness			
Test	1-4 Days	5-7 Days	8-10 Days	11-15 Days
Dick test	+ or ±	± or $\underline{\pm}$	$\underline{\pm}$ or –	–
Schultz-Charlton	–	– or ±	± or +	+

A definite negative Dick reaction during the first few days of the suspected attack of scarlet fever also makes the diagnosis doubtful. There is one exception, however; in the presence of an intense blush on the forearms as well as on the rest of the body, the so-called "boiled lobster rash," a moderately positive reaction may not stand out by contrast from the underlying rash and the reaction may appear negative. In some of these cases pressure on the forearm above the point of the test may make the reaction more visible.

The question will arise in many instances, when the diagnosis has been rendered doubtful by the result of the Dick test, whether it is justifiable to keep the patient in the hospital for the required thirty days. Patients who give a strongly positive reaction after admission into the scarlet fever ward can be protected after exposure by an injection of convalescent or horse antitoxic serum.

In Part B of Table 12 the relation between the Dick test and the Schultz-Charlton extinction test in scarlet fever is shown.

4. *Appearance of Old Positive Dick Reaction During Acute Stage of Scarlet Fever.*—One of the two patients shown in Table 11 (Verna B.) and another patient, a nurse, who also had given a strongly positive Dick reaction one week previous to developing scarlet fever, showed a most interesting and significant appearance of the original test at the time of the rash. The area on the forearm corresponding in size to the previous positive Dick reaction was very pale and had the typical blanched out appearance. This area was surrounded by a sharply defined ring of the scarlet fever rash, which was greatly intensified in redness when compared with the rest of the eruption on the forearm. This appearance persisted for a number of days until the rash faded. During the height of the eruption, a second Dick test was made over the site of the pale area and another one a little to one side. The reaction that developed within the pale area was very slight when compared with the second reaction beside it. Apparently, a certain amount of local cellular immunity had developed at the site of the original positive

Dick test. This immunity gave rise not only to the appearance of local pallor, which presented such a striking contrast to the rest of the blushed skin, but also to the slight positive reaction to the Dick test made over this pale area as compared with the second more strongly positive reaction on the adjacent skin. The ring of intensified rash making up the border that surrounded the pale area was probably due to the interaction between cells that were sensitized rather than protected by the minute antibody content within their substance and the toxin of the disease. These observations point to the probable local origin in the epidermis of some of the antitoxin production against scarlet fever.

One of the two patients had also received, a week previously, the initial immunizing dose of scarlet fever toxin. At the site of the injection, which was given subcutaneously in the arm, there appeared the same phenomenon during the time of the rash as was noted at the site of the Dick test. The area of pallor was larger, however, and the surrounding ring slightly wider.

5. *Group of Scarlet Fever Cases Showing Negative Dick Reactions During Convalescence (Regular Group).*—Table 13 shows twenty-seven scarlet fever patients who were Dick positive on admission to the hospital, and subsequently gave a negative reaction during convalescence from the disease. The reaction apparently becomes negative seven to ten days after the onset of the illness. In a few patients a shorter time was noted (from four to five days). The greater number of days before the appearance of a negative Dick reaction in the last three cases is accounted for by the longer intervals between the tests in these patients.

The period during convalescence when the patients show a negative Dick reaction corresponds to the appearance of antitoxic antibodies in their circulating blood (Table 9). This can be shown by the property of the serum to neutralize scarlet fever toxin and cause the blanching of an early rash in an acute case of scarlet fever (positive Schultz-Charlton test, Table 12).

The Dick reaction became negative a little earlier in convalescence, showing that it requires a certain concentration of antitoxic antibodies before the serum will cause a good blanching in an early rash. The Schultz-Charlton test as a quantitative measure for the antitoxin content is a much cruder method than the direct or indirect demonstration of the presence or absence of these antibodies by the Dick test. The table also shows that some of the patients developed a pseudoreaction during convalesence.

The results shown in Table 13 are certainly a striking corroboration of the statement of the Dicks that the reaction is positive during the early stages of scarlet fever and negative in convalescence. Two of the patients (Mollie D. and Seymour B.) had been tested at the Hebrew Orphan Asylum from one to two weeks previous to the attack of scarlet fever. Both of these children had given a + + positive Dick reaction. Four days after developing scarlet fever, Seymour B. gave a ± reaction; five days after the onset of the illness, Mollie D. gave a ± reaction. In both these children negative reactions were noted later on during the period of convalescence.

TABLE 13.

THE DICK TEST IN SCARLET FEVER PATIENTS DURING ACUTE AND CONVALESCENT STAGES OF THE DISEASE (REGULAR GROUP), WILLARD PARKER HOSPITAL

Name	Age, Years	Date of Admission	Days Ill Before Admission	Dick Test						Dick Test Negative Day of Illness
				First	Second	Third	Fourth	Fifth	Sixth	
Sophie B.	4	5/1	2	5/1 ±	5/3 —	5/10 —	5/17 —	....	....	4
Vincent C.	7	5/14	2	5/15 ±	5/17 —	....	....	....	....	5
Alfred G.	18	5/8	3	5/8 ±	5/10 —	5/17 —	....	....	....	5
Helen M.	8	4/30	2	5/1 ±	5/3 —	5/10 —	5/17 —	....	....	5
Tom R.	21	2/26	2	2/26 +	3/1 —	3/6 —	3/22 —	3/31 —	4/25 —	6
Jacob E.	11	5/6	1	5/0 ±	5/10 —	5/17 —	....	....	....	5
Rose T.	10	5/17	3	5/17 ±	5/20 —	....	....	....	....	6
Seymour B.*	13	4/8	4	4/9 ±	4/10 —	4/25 —	5/3 —	....	....	6
Mary O'H.	6	5/10	2	5/20 +	5/22 ±	5/24 —	....	....	....	7
Elizabeth G.	2	5/15	2	5/15 +	5/17 +	5/20 —	....	....	....	7
William F.	21	5/14	1	5/15 +	5/17 ±	5/20 —	....	....	....	7
Josephine M.	28	5/17	2	5/18 +	5/20 ±	5/22 —	....	....	....	7
Elias L.	9	4/29	3	4/29 +	5/3 —	5/10 —	5/17 — ps.	....	....	7
Mildred D.	9	5/10	3	5/20 +	5/22 ±	5/24 —	....	....	....	8
Mildred W.	1½	3/19	1	3/19 +	3/27 —	4/10 —	4/25 —	5/3 — ps.	5/17 — ps.	9
Edward P.	14	5/3	2	5/4 ±	5/10 —	5/17 —	....	....	....	9
Albert B.	7	5/12	1	5/12 +	5/17 ±	5/20 —	....	....	....	9
Paul B.	15	4/17	2	4/19 +	4/25 —	5/3 —	5/10 — ps.	....	....	10
Elizabeth G.	2	5/15	5	5/15 +	5/17 +	5/20 —	....	....	....	10
Margaret H.	41	4/23	3	4/24 +	4/25 +	5/3 — ps.**	5/10 — ps.	5/17 —	....	10
Nathan L.	11	5/12	2	5/12 +	5/17 ±	5/20 —	....	....	....	10
Julius C.	10	5/15	2	5/16 +	5/20 +	5/23 —	....	....	....	10
Germain B.	12	4/17	2	4/19 +	4/25 ±	5/3 —	5/10 —	....	....	10
George S.	26	4/12	4	4/4 ±	4/10 —	4/25 —	5/3 —	....	....	12
Catherine F.	14	4/24	4	4/24 ±	4/25 ±	5/3 —	5/10 — ps.	5/17 — ps.	....	13
Florence R.	1	4/26	1	4/30 +	5/ 3 ±	5/10 — ps.	5/17 — ps.	5/20 — ps.	5/22 — ps.	15
Mollie D.†	6	4/12	2	4/14 ±	4/25 —	5/3 —	5/10 —	....	....	13

* Had a ++ Dick test one week before attack of scarlet fever.
** Indicates negative-pseudo reaction.
† Had a ++ Dick test two weeks before attack of scarlet fever.

ACTIVE IMMUNIZATION WITH SCARLET FEVER TOXIN

We have injected more than 1,400 persons who gave a positive or a positive-combined Dick reaction with scarlatinal hemolytic streptococcus toxin. To avoid the constitutional symptoms noted by the Dicks, we begin with smaller doses and give three injections at weekly intervals instead of two injections at intervals of five days. The injections are made subcutaneously or intramuscularly.

We have found it convenient and helpful to interpret the dose of the toxin injected in terms of the skin test dose. In children under 12 years of age, we inject first 100 skin test doses, then 250 and again 250; for persons over 12 years of age, first 100, then 250 and 500 skin test doses are given. Recently we have been giving 500 skin test doses as the third dose to children over 3 years of age. For adults, 1,000 skin test doses may be used for the third injection. The toxin is diluted so that each cubic centimeter represents 500 skin test doses. The initial dose is 0.2 cc.; the second dose, 0.5 cc., and the third dose 0.5 or 1 cc., depending on the age of the person. When a large group is to be injected with the same dose, a dilution of the toxin is made of such strength that 1 cc. represents 100, 250, 500 skin test doses.

The reactions noted have been, as a rule, largely local and consist of a certain amount of redness and swelling at the site of injection. The second and third doses, although from two and one-half to five times as great as the first one, are followed, as a rule, by only very slight local reactions.

In a small proportion of the injected persons constitutional symptoms have been noted. In eight children and in one adult we have seen a scarlatiniform rash, which developed from twenty-four to forty-eight hours after the first injection of 100 skin test doses. There was a slight sore throat and elevation of temperature in these subjects, but no vomiting. The second injection in this group gave no constitutional and only slight or no local symptoms. The adult had a fine desquamation of the skin on the palms of the hands after the disappearance of the rash. She gave a negative Dick retest two weeks after the second injection.

Occasionally the second and even the third injection of toxin may be followed by a rash, slight sore throat and some febrile reaction. The symptoms disappear in from thirty-six to forty-eight hours. While these constitutional reactions occur only occasionally and are not serious, being seen in persons who are unusually susceptible to the effects of the toxin, yet it would seem desirable to avoid them. For that reason we are at the present time treating the purified toxin with formaldehyde according to the method suggested by Glenny and Hopkins for the production of toxoid from diphtheria toxin. This consists in adding 0.1 per cent. liquor formaldehydi to a toxin containing from 2 to 5 mg. of aminonitrogen per 10 cc., as determined by the Van Slyke method, and allowing the toxin to remain at incubator temperature for four or five weeks. In this way we aim to obtain a modified scarlet fever toxin or toxoid that will retain its immunizing value and can be used in larger doses without the risk of producing constitutional symptoms. This work is in progress, and the results will be reported as soon as they are finished.

The Dick retest, made from one to two months after the injections of the toxin, gave a great number of pseudoreactions, making quite difficult the interpretation of the developed immunity. A control test with heated toxin was found quite essential for reading the reactions at this time. With the purified toxin, the pseudoreactions in the retest can be largely avoided and the immunity results more accurately noted.

Table 15 shows the immunity results obtained with scarlet fever toxin in three institutions. The Dick retest was made from four to five weeks after the last toxin injection. At the Hebrew Orphan Asylum, 104 of the 143 injected children, or 72.7 per cent., gave a negative or pseudoreaction. At the New York Orphanage, forty-five out of ninety-one, or 49.4 per cent., gave negative or pseudoreactions. At the Leake and Watts Orphan Home, we injected the children with four doses of the toxin, consisting of 10, 25, 100 and 250 skin test doses. This institution was one of the first in which the testing and immunization against scarlet fever was carried out. We injected very small doses of the toxin at first, so as to avoid the more pronounced constitutional reactions noted by the Dicks with larger doses of the toxin. At this institution, eighteen of the forty children retested, or 45 per cent., had negative reactions or pseudoreactions.

TABLE 15.

IMMUNITY RESULTS WITH SCARLET FEVER TOXIN

Doses given patients under 12 years: 100, 250 and 250 skin test doses at weekly intervals

Doses given patients over 12 years: 100, 250 and 500 skin test doses at weekly intervals

Dick retest after four to five weeks

Institution	Per Cent Positive Dick at Original Test	Total Re-tested	Dick Positive and Combined			Dick Negative and Pseudo	
			Number Positive As in Original Test	Number Less Strongly Positive	Per Cent. Less Strongly Positive	Number	Per Cent.
Hebrew Orphan Asylum	29.2	143	19	20	14.0	104	72.7
New York Orphanage	44.4	91	10	36	39.5	45	49.4
Leake and Watts Home	22.0	40	12	10	25.0	18	45.0
Total		274	41	66	24.0	167	61.0

Of the 274 children retested in the three institutions 167 or 61 per cent, showed that they had become immune. Most of the reactions noted were pseudoreactions. The large number of pseudoreactions at the retest indicates that a certain amount of protein sensitiveness deveolped after the toxin injections.

Conclusions

1. The Dick test is a reliable index of immunity and susceptibility to scarlet fever.

2. In conjunction with active immunization with scarlet fever toxin, it will help in solving the problem of the control of scarlet fever.

3. It serves to indicate the susceptible persons who need immediate passive immunization with scarlet fever antitoxin. The possibility of interpret-

ing the reaction within twelve hours is of great clinical value for this purpose. In our experience up to the present time seven of the positive reactors and none of the negative reactors have developed scarlet fever.

4. The Dick test helps in the diagnosis of doubtful cases of scarlet fever. A strongly positive reaction early in the disease and again late in convalesence speaks against the diagnosis of scarlet fever. A definite negative reaction during the first two days of rash should also put one on guard that he may not be dealing with scarlet fever. A negative reaction does not usually develop until the sixth to the tenth day of the disease, at a time when the rash has faded. Two children who had a strong positive reaction on admission to the hospital developed scarlet fever five days later. Both patients gave a negative reaction during convalescence.

5. Other applications of the skin test consist in studying the specific character of the toxin produced by different strains of hemolytic streptococcus. Such studies would be of value in the diagnosis of doubtful cases of scarlet fever, in the classification of clinical conditions caused by the hemolytic streptococcus but not associated with scarlatiniform rashes, and in the identification of normal and persistent carriers of the specific streptococcus of scarlet fever. Patients convalescent from scarlet fever may even be required to be free from the specific organisms before they are discharged from quarantine, if a method of accurate and rapid bacteriologic diagnosis can be established.

6. A new impulse will be given by the application of the test to the study of scarlet fever to determine whether the disease includes a single clinical entity or whether, as was long suspected, the clinical conception of scarlet fever includes a variety of diseases with similar symptoms, but not necessarily associated with the presence of the specific strains of the hemolytic streptococcus of scarlet fever.

7. Scarlet fever is a combined toxic and bacterial infection, caused by a specific hemolytic streptococcus. The immunity following an attack of scarlet fever is antitoxic and not to any extent antibacterial. This is shown by the fact that convalescent patients, who give a negative Dick reaction, may develop secondary septic complications produced by the specific streptococcus. A permanent antitoxic immunity following the disease explains the freedom from second attacks of clinical scarlet fever. It is possible, however, that subsequent infections without a rash caused by the specific streptococcus may occur in those who have recovered from an attack of scarlet fever. Such infections may also occur in those who give a negative Dick reaction.

8. The diluted toxin keeps well, and can be distributed in this form ready for the test. A control test, consisting of toxin heated at boiling temperature in a water bath for one hour, has been suggested to identify the pseudoreactions.

9. The results with the Dick test in normal persons indicate that the percentage of susceptible persons by age groups and by social status resembles closely the similar percentage of persons susceptible to diphtheria noted with the Schick test.

10. The application of the Dick test in the early stage of scarlet fever showed a positive reaction in each one of the 141 patients tested. The reactions rapidly became less strongly positive and were found negative from the seventh to the tenth day of the disease.

11. Of 170 scarlet fever patients tested during convalescence 158, or 93 per cent., gave a negative Dick reaction. There is some doubt about the diagnosis of scarlet fever in most of the remaining twelve patients, who

showed a persistent positive reaction in convalescence. Positive reactions can be brought out in scarlet fever convalescence by using a stronger dilution of the toxin (1:100). It is therefore necessary, in making comparative studies, to adhere to one standard dilution of the toxin.

12. Active immunization with scarlet fever toxin is a safe procedure, and is not to any extent associated with the development of constitutional symptoms, if the dose of the toxin is gradually increased. The amount is most conveniently increased by the skin test dose, the quantity injected representing 100, 250 and 250 skin test doses for children under 12 years, and 100, 250 and 500 skin test doses for persons over 12 years of age. The injections are given one week apart. The immunity results as shown by the Dick retest seem to be encouraging. A large proportion of the selected individuals show a pseudoreaction, indicating the necessity of adding the control test to the Dick test in checking up on the immunity results with scarlet fever toxin.

13. Purification of the toxin by the sodium chlorid and acetic acid precipitation method gives a preparation that is better for purposes of the test and active immunization, as it eliminates to a large extent the proteins causing the pseudo element in the Dick test and the local reactions, as well as the sensitization after the immunizing injections.

14. Treating the toxin with formaldehyde, to deprive it of nearly all its toxic properties without impairing its antigenic value, seems to be a possible further step in the development of the process of active immunization against scarlet fever.

15. The scarlet fever toxin is neutralized in multiple proportions by the antitoxic serum.

For bibliography see the original article.

ANTISCARLATINAL SERUM OF DUAL POTENCY

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W. H. Park and A. W. Williams

The question of the complex nature of hemolytic streptococcus antiserum has been studied since the time of Marmoreck,[1] Van deVelde[2] and others.

Protective power was early demonstrated. Antitoxic powers were also demonstrated broadly for the scarlet fever strains by Moser,[3] Savchenko[4] and others and were recently made clear cut by the work of Dochez[5] and of the Dicks.[6]

Realizing that a serum having the power to protect against the living culture, as well as to neutralize the toxin, might have advantages over a strictly antitoxic serum, we have been inoculating several of our horses by what must be called a modified Moser method. The modifications have been learned by our work on diphtheria antitoxin and antipneumococcus serum, that is, that a portective serum is obtained most easily by the repeated inoculation of the whole killed culture into the vein, and that the antitoxin serum is best obtained by the repeated inoculation of toxin subcutaneously.

We inoculated the killed whole washed cultures intravenously, and the Buchner filtrates of a six day growth in blood bouillon subcutaneously. Cultures of the filtrates showed 100 colonies of the streptococcus to the cc.

One of the horses which we inoculated in this way, No. 86. has been under treatment for nine months; the others for much shorter times.

The inoculations into this horse were started on January 15th, 1925, by giving the whole centrifuged 24-hour broth cultures intravenously. Two days later the Buchner filtrate of a six-day blood broth culture was started subcutaneously. The doses were continued on alternate days and were gradually increased. In about three months the serum began to show blanching in the Schultz-Charlton test. In five months the serum was strongly blanching, and 1 cc. neutralized 10,000 Dick skin test doses of the streptococcus toxin. At this time it showed no definite protection. After nine months 1 cc. neutralized 40,000 skin doses of toxin, and 0.05 cc. protected mice against 100 M.L.D. of very virulent living streptococci.

The clinical results with this serum have been excellent but it is impossible yet to state that they have been better than those obtained with antitoxic serum alone. Theoretically such a dual serum should not only neutralize the toxic effects of the streptococcus in scarlet fever but should reduce the number of so-called complications and sequellae of this disease.

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COMPLEXITY OF THE SCARLET FEVER TOXIN AND ANTITOXIN

A PRELIMINARY REPORT

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While we were carrying out the tests upon which this communication is based Kirkbride and Wheeler[1] reported that a person had been found who was susceptible to the Dochez toxin but who was not susceptible to the Dick or Williams toxin and that this person's serum neutralized the Dochez toxin in two persons but did not do so in the donor of the serum. The work of Williams had already shown not only that from certain cases of erysipelas, infected wounds and normal throats, hemolytic streptococci could be isolated that produced toxins similar to those isolated from the great majority of cases of scarlet fever, but that other strains from these sources produced toxins that varied in a greater or less degree from those produced by the ordinary scarlet fever streptococci.

In testing for the potency of scarlet fever antitoxin we noticed that an amount of horse antitoxic serum which would neutralize the toxin in a majority of children would totally fail in others. It was also noted that the antitoxic serum of different horses would occasionally vary in the ability to neutralize a toxin in the same child and finally that in some individuals in

TABLE 1

A preparation of horse antitoxin which neutralizes Dick toxin in some children and not in others

Child	Result of Dick Test	Result of Serum control	0.05 cc. Serum 1:25. Horse 35 + 5 Skin Doses Toxin	0.05 cc. Serum 1:25. Horse 39 + 5 Skin Doses Toxin
1	+	—	++	++
2	+	—	—	—

Neutralizing value of antitoxin from different horses in the same individuals

Cases	Result Dick Test (1 Skin Dose)	0.05 cc. Serum 1:25 + 5 Skin Doses Toxin		0.05 cc. Serum 1:25 + 5 Skin Doses Toxin	
		Horse 87	Horse 32	Horse 7	Horse 8
3	+	++	++	—	—
4	+	+	+	—	—
5	+	—	—	—	—

An example of a child in which the usual amount of antitoxin does not neutralise the dose of toxin but a multiple dose does

Case	Result Dick Test 1 Skin Dose	1:10 Dilution	1:100 Dilution
6	+	++	++
7	+	—	++

whom the ordinary amount of antitoxin was not effective an increased amount of antitoxin was.

These observations by Kirkbride, Williams and ourselves seemed to prove that the scarlet fever toxic filtrate produced by a single strain was not a single toxin but a group of toxins and that a person might be immune to one or more of the component toxins of a toxic broth and yet susceptible to others and that certain horses react in producing antitoxin more to one of the components and certain horses to another in much the same way as Park has shown that of two horses receiving simultaneously the same quantity of tetanus and diphtheria toxins one may develop a potent diphtheria antitoxin and a weak tetanus antitoxin while the other will do the opposite. The same difference is noted in the amount of specific and group agglutinins which develop in different animals receiving the same dose of bacterial antigen.

Our interest was aroused by these observations and we decided to note carefully the results which followed mixing individual serums from convalescent scarlet fever patients with the Williams toxin and testing the mixtures on the skin of many children. One group of 26 Dick positive children which showed no serum or protein reaction gave the results (of the test) shown in table 2.

TABLE 2

Number of Child	Dick Test 5 Skin Doses	0.01 cc. Convalescent Serum A + 5 Skin Doses	0.1 cc. Convalescent Serum A + 5 Skin Doses	0.01 cc. Convalescent Serum B + 5 Skin Doses	0.1 cc. Convalescent Serum B + 5 Skin Doses
1-13	++	—	—	—	—
14	++	±	—	+1	0
15	+	+	0	±	0
16	+	+	0	—	0
17	+1	+1	0	±	0
18-19	++	++	++	+	—
20-26	++	++	++	±	—

— = negative reaction; 0 = no test made; + = moderate reaction; +1 = a somewhat stronger reaction: ++ = marked reaction.

TABLE 3

Skin Reaction—Scarlet Toxin, 0.1 cc. of 1:4000	Skin Reaction—Erysipelas Toxin, 0.1 cc. of 1:100	Skin Reaction—Scarlet Toxin, 0.05 cc. 1:2000; Erysipelas Toxin, 0.05 cc. 1:50
+1	+	+1
++	++	++
++	+	+1

+ = moderate reaction; ++ = a marked reaction.

Horse antitoxin stimulated by the Williams strain acted like convalescent serum B, and completely neutralized this toxin as shown by the Dick test in the last nine children. In a series of routine tests carried out by Schroder, Williams and Park we noted that a certain dose of an individual convalescent serum failed to neutralize the Williams toxin in 35 but did neutralize it in 218 non-immunes. An interesting point was to explain how a partially neutralized toxin produced as marked a skin reaction as it did before the addition of the serum. A test made by combining a skin test dose of erysipelas toxin with one of scarlet toxin and injecting it intracutaneously revealed the interesting fact that adding together the skin test dose of the two toxins and injecting the combined dose intracutaneously did not increase the size or severity of the skin reaction.

Summary and Conclusions

The fact that an individual convalescent scarlet fever serum neutralizes the toxin of a certain scarlet fever streptococcus in certain susceptible persons and not in others proves that the toxic filtrate has more than one toxin and that a person may be susceptible to one component and not to another or others. We can conceive that the usual scarlet fever strain makes a toxic filtrate containing the different toxins, A, B, C, D; a less common strain A, C, D, E or B, C, D, E; strains from some septic sore throats C, D, H, I and from some erysipelas cases C, D, K, L. Antitoxins to a greater or less degree would develop in any person or animal injected with any toxic filtrate to neutralize the actual toxins present in the filtrate.

The finding that a certain convalescent serum or antitoxic horse serum will fail to neutralize a toxin for certain persons in the same quantity which is successful in others, but in larger amounts succeeds, proves that the antitoxic response is different for the different component toxins either because of the different proportions of the toxins in the filtrate or because the child or animal responds differently in degree to the stimulus of the different toxins. We have the analogy of horses receiving the same amount of diphtheria and tetanus toxin. Some produce strong antitoxin for diphtheria but weak for tetanus and others strong antitoxin for tetanus but weak antitoxin for diphtheria.

A convalescent scarlet fever serum from a single patient may fail to cause blanching in a true case of scarlet fever because the two cases are due to two strains that differ in at least one toxin and so in one induced antitoxin. It is also probable that there are strains of streptococci that have the power to produce scarlet rash producing toxins which are quite different from the standard Dick strains, so that after convalescing from infections due to one strain a second infection might occur from the other strain. Every year a few cases sent into the hospital with the diagnosis of scarlet fever, after recovery develop a second attack. We have been inclined to think that the first attack was not scarlet fever while, if we had made a culture, we possibly would have found a strain of streptococci that made different toxins from the usual cases. If this assumption is true a case of scarlet fever at the onset might give a negative Dick test to the Dick strain toxin and its serum would then neutralize the standard toxin and blanch the rash of an ordinary case of scarlet fever. The point is that it is not safe at the present time to limit the diagnosis of scarlet fever to cases due to the usual strains making the standard group of toxins. At most the exceptional strains cause only a small minority of the cases of scarlet fever. Cases that do not respond to the serum should be studied carefully to determine whether their strains are typical ones. It is possible that in these a polyvalent antitoxin serum is indicated or it may simply be that the scarlet fever streptococci or other streptococci or other micro-organisms have invaded the deeper tissues and are beyond the help of antitoxins. Further investigation is necessary to determine the value of a bactericidal serum in these cases.

Reference

[1] Kirkbride and Wheeler: Proc. Soc. Exper. Biol. and Med., November, 1924, 22, 85.

HEMOLYTIC STREPTOCOCCI IN SCARLET FEVER AND ERYSIPELAS (SEROLOGIC RELATIONSHIPS)

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By Anna W. Williams and Caroline R. Gurley
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Summary of Work

More than 300 case strains of hemolytic streptococci from scarlatina, erysipelas and other sources have been studied in whole or in part as to their serologic relationships. A more intensive comparative study has been made of about 150 of these strains, the results from which are summarized in two tables.

Some strains have given evidence of splitting into coarse and fine (rough and smooth) colonies, subcultures from which may give agglutinative reactions differing from the original strain. This phenomenon could explain at least some of the irregularities reported in studies of this group of bacteria.

The table shows the following results:

1. (a) From early stages of scarlatinal cases strains of hemolytic streptococci have been obtained, most of which by absorption of agglutinins fall into one of three groups and one sub-group.

(b) Accepting Griffith's Type I and Type II as distinct agglutinative groups we describe four agglutinative types and one sub-type. Type I—the same as Griffith's Type I, our type strain is $2.^2$ Only 4 strains of ours fell in this group. Including Griffith's type strain we have five strains from scarlatina and one from mastoiditis in this group. It is dominant over our strains that fall in our 55^4 group which we had earlier designated as Type I.

Type sub I. Our type strain is 55^4. Most of our strains fall in this group. As Griffith says that our 55^4 falls in his Type III group, his Type III must be considered Type Sub. I. Two Dochez strains and two Dick strains sent to us belong here.

Type II. Working with Griffith's Type II strain we have found no strain in New York City that falls in this group.

Type III. All of our scarlatina strains that belong to anginosus by carbohydrate fermentation—9 strains—fall together by absorption of agglutinins. 57^2 is the type strain of this group.

Type IV. A comparatively large group of strains—17—are of this type. The most important fact concerning this group is that its members cross absorb with strains of Group I,—16 strains—from erysipelas. The type strains is 28^1.

Strains of hemolytic streptococci from erysipelas fall into one large group, Type I, a small group, Type II and a few strains not yet grouped. Most of Birkaug's strains fall into the larger group. The 3 strains of Type II belong to the subacidus group by carbohydrate fermentation.

Strains from all of these groups both from scarlatina and from erysipelas produce a toxin that may be neutralized in some human beings by both scarlatinal convalescent serum and by our Type sub. I antitoxic horse serum.

TABLE I

RELATIONSHIP OF STRAINS OF HEMOLYTIC STREPTOCOCCI TO CARBOHYDRATE REACTIONS AND AGGLUTININ ABSORPTIONS

		Carbohydrate Reactions					Agglutinin Absorptions								
Source	No. of Cases	Lactose	Manitol	Salicin	Inulin	Holman's Types	Type I (22)	Type Sub. I (55)	Type II Grif.	Type III (57)	Type IV (28)		Type Ery. I (C^3)	Type Ery. II (B^3)	No type yet found
Scarlatina	77	+	—	+	—	Pyogenes	5	38	1	..	17	=	(17)	..	16
Scarlatine	9	+	—	—	—	Anginosus	..	..	..	9	..		..	..	..
Scarlatina	3	—	+	+	—	Infrequens	..	..	..	..	..		..	..	3
Erysipelas	22	+	—	+	—	Pyogenes	..	..	..	..	(16)	=	16	..	6
Erysipelas	4	—	—	—	—	Subacidus	..	..	..	..	..		..	4	..
Other sources	16	+	—	+	—	Pyogenes	1	3	..	..	1	=	(1)	..	11

A type sub. I horse antiserum which is strongly protective against the subtype I strains (whole culture), has not protected against the whole culture of the type strain 28[1] of Type IV group.

CONCLUSIONS

Scarlatinal rash is merely a symptom produced by the toxins[1] of a large group of hemolytic streptococci, certain members of which may cause erysipelas. These members, while producing a toxin similar to that of all strains from scarlatina, may stimulate the production of an antiserum against the whole culture different from that produced by strains belonging to other types. Erysipelas, therefore, may require a special *antibacterial* serum while any type of antitoxic serum (stimulated by scarlatinal or other hemolytic streptococcal exotoxin) may combat the exotoxic element in at least certain cases of this disease.

THE PURIFICATION OF THE STREPTOCOCCUS ANTITOXIN FOR SCARLET FEVER

Edwin J. Banzhaf

The method herein given is the same as used by us in experimental purification of diphtheria and tetanus antitoxins since 1912.

To the citrated plasma 0.8% of an equal volume of trikresol and ether is added. A half volume of water is then used to dilute the trikresolized plasma. Saturated solution of ammonium sulphate is added up to 25%. Thus to every 75 cc. of diluted plasma 25 cc. of saturated solution ammonium sulphate is required. A slight precipitation is observed at this point. The whole is heated rapidly up to 57° C. and held at this temperature for one and one-half hours. It is then removed and filtered hot. During the heating, considerable more precipitation is formed. This precipitate, which contains only the antitoxin held in the remaining fluid of the moist precipitate, can be discarded or it can be taken up with water and reprecipitated with ammonium sulphate and the resulting filtrate added to the first.

The clear filtrate from the heated mixture is brought up to 46% saturated solution ammonium sulphate. This will precipitate all the antitoxin, and after standing over night is filtered through hardened papers or muslin cloths. Press to remove all excess of fluid and dialyze in parchment bags in running water. Dialysis is continued for eight days or until the solution is practically free from ammonium sulphate. After dialysis 0.8% salt is added and 0.8% of an equal volume of trikresol and ether. It is then stored in a cold room for at least two weeks, preferably a month. It can then be pulped and Berkefeld filtered.

CONVALESCENT WHOLE BLOOD, PLASMA AND SERUM IN PROPHYLAXIS OF MEASLES

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Abraham Zingher

The use of convalescent serum in the prophylaxis of measles was a logical sequel to the application of human convalescent serums in preventing and curing other infectious diseases for which the causative agents, as in measles, have not yet been definitely isolated and identified. Scarlet fever and poliomyelitis are additional examples of infections in which the serum of convalescents and of recovered patients has been successfully used in prophylaxis as well as in therapy.

The serums of adults who have had these infections in childhood also contain antibodies, although they are present in a lower concentration. Such antibodies are transmitted from the mother to the offspring through the placenta. This is shown by the temporary protection of many infants under 6 months of age against different infectious diseases, such as measles, scarlet fever and poliomyelitis. In the transmitted immunity to diphtheria there is even a close quantitative parallelism in the antitoxin content of the mother's blood and that of the new-born infant.

The presence of these protective antibodies in the parents' serum is extremely important, as it enables us to utilize a source of serum supply that is universal and readily available, whereas with convalescent serum we are dependent on a limited number of donors. In this connection it may be mentioned that the Writer strongly recommended, during the epidemic of poliomyelitis in 1916, that the blood serum obtained from parents be injected in doses of from 30 to 60 cc. into young and susceptible children, so as to reestablish a temporary passive immunity during the period of epidemic.

Historical

The first published results on the use of convalescent measles serum were by Nicolle and Conseil, and appeared in 1918. These observers in 1916 succeeded in protecting a child after exposure to measles. In 1916, Park and Zingher injected forty-eight exposed children with convalescent measles serum at the Metropolitan Hospital on Welfare Island. Twenty-eight of these children received doses varying from 1.5 to 4 cc. and twenty received doses of 8 cc. In the first group, six children developed measles; one, two days; one, seven days; one, eight days; one, fifteen days; one seventeen days, and one, twenty-five days after the serum injection. In the second group, none developed measles. These results were not published.

In 1918, Richardson and Connor of Boston reported the successful use of convalescent serum in a small group of exposed children. In 1919 Degkwitz, working in Pfaundler's clinic in Munich, began a systematic series of observations with convalescent serum in the prophylaxis of measles. He subsequently organized the work on a broad scale in Munich and up to the present time has injected more than 1,000 children with convalescent serum. He was followed by various observers in Germany, Hungary, Italy, Spain, South America and the United States.

TABLE 1.

DEATHS FROM MEASLES IN NEW YORK CITY FOR YEAR 1922

Age in Years	Total Deaths	Total Deaths in Each Group	Per Cent. of Total Deaths in Each Group	Per Cent. of Deaths Under 5 Years
Under 1	236			
From 1 to 2	410			
Total		646	66.1	93.4
From 2 to 3	160			
From 3 to 4	64			
From 4 to 5	43			
Total		267	27.3	
From 5 to 9	54			
10 and over	10			
Total		64	6.6	
Total		977		

Total deaths from Jan. 1 to July 30, 1922	935	(95.7%)
Total deaths from July 1 to Dec. 31, 1922	42	(4.3%)

Donors and Methods of Obtaining Blood

The donors should be healthy persons, preferably adults, who are free from syphilis and tuberculosis. When adult donors are not available, it may be justified to take, under special conditions, a small amount of blood from children over 10 years of age. It is difficult, however, to see the advisability of the recommendation made by Degkwitz, that as much as 60 cc. of blood should be taken from children under the age of 5 years. I am afraid that bleeding such young children will get this method sooner or later into disrepute. Parents might be willing to allow a small amount of blood to be taken from convalescent children or from brothers and sisters who have had measles previously, for use in the same family; but most parents would decidedly object to having children under 5 years or even under 10 years of age bled for general serum distribution.

From adult donors, about 500 or 600 cc. of blood could be readily obtained. A second bleeding from the same donor could be obtained two or three weeks later, if further observations show that these late serums are fairly equivalent to convalescent serums in concentration of antibodies. Suitable compensation should be made to the donor for the blood withdrawn.

The blood should be collected under sterile precautions by allowing it to flow into a 500 or 1,000 cc. bottle. The container is closed with a double perforated stopcock, through which pass two pieces of glass tubing. To one is attached a piece of rubber tubing and a large bore (16 gage) needle, similar to those generally used in blood transfusions. The other piece of glass tubing is plugged with a little cotton, and to it is attached a piece of rubber tubing, which serves the purpose of producing a partial vacuum in the bottle by means of suction. The entire apparatus should be wrapped and sterilized. The blood can be collected and allowed to clot, and the serum subsequently drawn off, or it may be kept fluid by the addition of 0.5 per cent. sodium citrate and the supernatant plasma afterward decanted. One volume of 5 per cent. solution of sodium citrate to every nine volumes of blood is previously placed in the bottle. The decanted serum or plasma is subsequently tested for sterility, pooled with the serums of two other donors, and divided into individual ampules containing 3 cc. Degkwitz considers 2.5 cc. as one immunizing unit. For the better preservation of the serum, it has been recommended to dry it in the ampules in vacuo, and then dissolve the residue with salt solution, before use. The fluid serum has been found to retain its protective properties for six months.

OPTIMUM TIME FOR BLEEDING DONORS; POOLED SERUM

According to Degkwitz, the best time for bleeding convalescent donors is from the sixth to the tenth day after the temperature has reached normal. Blood serum obtained from persons who recovered from measles several months or even several years previously has also been used with success. The dose, however, should be larger.

The Wassermann test is made on the serums unless the donors have been previously tested. Three different serums should be pooled. It is possible that an occasional convalescent donor may be a poor antibody producer, just as certain horses are poor antitoxin producers; the pooling, however, will minimize the danger of using ineffective serums.

DOSE OF SERUM AND PERIOD OF EXPOSURE

The dose recommended by Degkwitz is 2.5 cc., or 1 immunizing unit, for children who have been exposed for not more than four days; 5 cc., or 2 units, for children exposed for from five to six days, and 7.5 cc., or 3 units, for children exposed seven days to infection with the measles virus. According to the same observer, the injection of serum on the eighth day of exposure and later will not prevent the development of the disease, nor will it modify the character of the symptoms. Our own experience and that of some of the French observers does not bear out this statement. The disease can be prevented in some of the patients, and is distinctly modified in some of the others, when the serum is injected as late as the eighth or ninth day of exposure. The serum is injected intramuscularly into the arm, thigh or gluteal region.

The dose recommended is for a child 3 years of age. For older children the dose should be in proportion to the age and weight of the child.

The period of exposure is calculated as follows: When the exposed children have been constantly in contact with the measles patient and the latter is seen on the first day of rash, the period of exposure is counted as four days; if seen on the second day of rash, as five days, etc. If the patient is seen at the time of the appearance of Koplik's spots and no rash, the exposure of the contacts is three days. Occasionally a patient is brought into a hospital and the rash appears on the following day or two days later. The exposure of the contacts will be one or two days.

COMPLETE PASSIVE IMMUNIZATION

There are distinct indications for attempting to produce complete passive immunization by increasing doses of serum. As thorough and as complete protection as possible should be given to many children who are very young or feeble; children suffering from rickets, tuberculosis, diphtheria or whooping cough; those convalescing from other infectious diseases, and children who would be in immediate danger from an attack of measles, such as undernourished patients or feeding infants. In institutions, also, and in hospitals, where the paramount indication is to stamp out an outbreak of measles, the early and prompt administration of convalescent serum is strongly indicated and will often prove a life saving boon. The colder seasons of the year, with the greater danger of respiratory complications, will also often be found an indication for complete immunization.

Modified Mild Measles

In private practice, when we are dealing with normal and healthy children, special conditions may also indicate the advisability of producing a complete but temporary protection.

Every clinician realizes, however, that it would be much better under ordinary conditions if our efforts should result in a permanent immunity of the children with the least risk involved. Such a permanent protection can be obtained by an attack of measles. If the disease can be modified, however, so that a mild attack of measles will develop, we shall have accomplished our aim. Such modified measles can often be produced if children are injected from the fifth to the tenth day after exposure with 2.5 cc. of convalescent serum, or with 5 cc. of serum from brothers and sisters who have recovered from measles some time previously, or with 10 cc. of adult serum. The principle involved in this method has been recommended by Debré and Ravina, and it would appear to be a more reasonable and logical procedure in private practice than the attempt to produce a complete but short immunization. My own observations also indicate that a peculiarly modified, mild form of measles can be obtained when small doses of convalescent serum are injected as late as the eighth day of exposure.

The modified form of measles is characterized by a moderate rise of temperature lasting for one or two days, and by a curious rash, scarcely resembling ordinary measles, which is scattered over face and body, and to a less extent over the extremities. The individual macules are about the size of the head of a pin, occasionally surmounted by a tiny vesicle. At times there are larger macules scattered between the small ones. Conjunctivitis, coryza and Koplik's spots are frequently absent; when present, these symptoms are very mild. The clinical picture is one of a mild form of measles as compared with the type usually seen. According to various observers, complications are very rare. In the group of cases that came under the Writer's observation, no complications were noted. Degkwitz also states that the modified form is not very infectious. This is probably on account of the slight or absent catarrhal symptoms with the lessened possibility of droplet infection.

Convalescent serum, if always available, would be most suitable for the purpose of modifying the attack of measles. Practically, however, convalescent serum will frequently not be obtainable. Under all conditions, the blood of brothers and sisters or of parents can nearly always be obtained. The serum or plasma does not have to be first separated and then injected. With an ordinary 10 or 20 Record or Luer syringe the blood is simply withdrawn and immediately injected intramuscularly. To enable the physician to work without haste, from 1 to 2 c.c. of a 5 per cent. solution of sodium citrate can be drawn into the syringe before the blood is taken from the donor. The whole blood is injected into the thigh muscles or into the gluteal region according to the intramuscular method that the Author recommended for the treatment of scarlet fever with convalescent whole blood. When whole blood is used, instead of serum, double the amount recommended for serum should be injected.

DURATION OF IMMUNITY CONFERRED BY CONVALESCENT SERUM

The duration of passive immunity produced by injecting convalescent serum in the doses recommended during the first few days after exposure is rather short, not lasting more than from four to five weeks. When convalescent serum is injected on the sixth day or later, a mixed type of immunity is produced, which lasts for from two to three months. If a modified attack of measles develops, the immunity is probably permanent.

The reports of different observers indicate that the passive immunity will last only from thirty to forty-five days. Following a second exposure, one of our patients developed typical measles forty-two days after a protective dose of serum.

Table 2 gives the dosage of convalescent serum, or serum from recovered patients (brothers and sisters) and from adults. It also indicates the type of immunity in which the protection is complete, and the type of disease in which the protection is incomplete.

CENTRAL SERUM STATIONS

The New York City Department of Health is offering at the present time to practicing physicians of the city a limited amount of convalescent plasma in individual vials containing 3 and 5 cc. A request is made that the physicians report to the department careful observations of the results obtained.

PERSONAL OBSERVATIONS WITH CONVALESCENT WHOLE BLOOD AND SERUM

The ease and simplicity of using citrated whole blood in hospital work first induced the Writer to immunize the exposed children in this way. The more recent work was carried out with convalescent serum or plasma.

The donors were from five to fifteen days convalescent patients. In some instances we used the blood of individual donors; in others, the pooled blood, plasma or serum of several donors.

The total number of exposed persons injected was 160. Tables 3 and 4 give a summary of the different groups. Each group represents one or more individuals, who were exposed at the same time either in hospital wards at the Willard Parker Hospital, in institutions or in private homes.

TABLE 2

Measles Prophylaxis

Days of exposure		1	2	3	4	5	6	7	8	9	10	11	12
Onset of clinical symptoms in measles		Catarrhal		Kop-lik spots	1st day of rash								
Amount of serum for complete protection	Convalescent	2.5 to 3 c.c.				5 c.c.		7.5 c.c.					
	Recovered cases (brothers and sisters)	5 to 6 c.c.				7.5 c.c.		10 c.c.					
	Adults (parents)	10 c.c.				15 c.c.		20 c.c.					
Type of immunity in complete protection		Passive				Mixed				None			
Amount of serum for incomplete protection	Convalescent	1.5 c.c.					2.5 c.c.						
	Recovered cases (brothers and sisters)	2.5 c.c.					5 c.c.						
	Adults (parents)	5 c.c.					10 c.c.						
Type of measles in incomplete protection		Modified										Typical	

GROUPS OF CASES IN WHICH MEASLES CONVALESCENT BLOOD, PLASMA OR SERUM WAS INJECTED

GROUP 1.—Five children were exposed in the diphtheria pavillion, May 24. Eight days later each child was injected with 20 cc. of convalescent whole blood from a donor five days convalescent. Two of the five children developed modified measles, one ten days after receiving the injection and after an incubation period of eighteen days; the other one fourteen days after the injection and after an incubation period of twenty-two days. The rash consisted of a few scattered macules, the size of the head of a pin. In the first child the rash was limited to the face; in the second one it was also present on the trunk, but not on the extremities. Neither one of the children showed catarrhal symptoms, conjunctivitis or Koplik's spots. There was slight elevation of temperature for two days. The clinical picture was that of a mild atypical form of the disease, which could not have been identified as measles except for the history of exposure. These cases indicate that measles developing in children who receive convalscent serum eight days or more after exposure may show a modified form. The other three children of this group were protected. One child, aged 3 years, exposed at the same time, received no convalescent blood, and developed typical measles fifteen days after exposure.

GROUP 2.—This child was in the same cubicle with the control just mentioned. She was injected on the day after the rash appeared (the fifth day of exposure) and was protected.

GROUPS 3 and 4.—Ten children were exposed in one of the scarlet fever wards. May 2, the fourth day of exposure, eight of the ten children, varying in age between 2 and 5 years, were each injected with 20 cc. of convalescent blood. These children were protected. The supply of convalescent blood being exhausted, the remaining two children were not injected until May 5, the seventh day of exposure. One of these a child, aged 4, an older brother of a boy, aged 2, injected in the group of eight children, developed a typical rash on May 25, twenty days after the injection of blood and twenty-seven days after exposure. He had a modified rash and mild catarrhal symptoms.

GROUP 5.—Four children were exposed in one of the wards of the reception hospital. Each of the children was injected with 20 cc. of convalescent blood on the seventh day of exposure. No measles developed. A fifth child, aged 5 years, was not injected on account of a questionable history of measles, and developed a typical measles attack on the thirteenth day of exposure.

GROUP 6.—Four children were exposed in one of the diphtheria wards to a patient with measles who developed a rash on the day after admission to the hospital. On the following day, the second day of exposure, each of the four children was injected with 20 cc. of convalescent blood. No measles developed.

GROUP 7.—This child was in a separate room in the scarlet fever pavilion and was exposed to a second child who developed a typical measles rash four days after admission to the hospital. Twenty cubic centimeters of convalescent blood was injected and the child was protected.

GROUP 8.—A child aged 2 years, injected on the eighth day of exposure with 20 c.c. convalescent blood, was protected. The child was in a separate room in the scarlet fever pavilion, and was exposed to a patient who had developed a measles rash four days previously.

TABLE 3.

CONVALESCENT WHOLE BLOOD, PLASMA AND SERUM IN THE PROPHYLAXIS OF MEASLES

	Patients Injected								Controls	
Group	Number Injected	Age, Years	Convalescent Whole Blood	Convalescent Serum or Plasma	Days of Exposure	Number Developed Measles	Period of Incubation	Type of Rash	Total Number and Age	Number That Developed Measles Period of Incubation and Type
1	5	2, 2, 3, 5, 6	20 c.c.	......	8	2	18 days 22 days	Moderate	1 (3 yrs.)	1 (15 days) Typical
2	1	1½	20 c.c.	......	5	0	......	....	....	....
3	8	2, 2, 2, 2½, 2½, 4, 5, 5	20 c.c.	......	4	0	......	....	....	....
4	2	4, 4	20 c.c.	......	7	1	27 days	Typical	....	....
5	4	2, 2½, 2½, 5	20 c.c	......	7	0	......	....	1 (5 yrs.)	1 (13 days) Typical
6	4	1½, 2, 3, 4	20 c.c.	......	2	0	......	....	....	....
7	1	3	20 c.c.	......	4	0	......	....	....	....
8	1	2	20 c.c.	......	8	0	......	....	....	....
9	2	3, 6	10 c.c.	......	3	0	......	....	....	....
10	2	22, 25	15 c.c.	......	6	0		....	....	....
11	1	1½	10 c.c.	......	4	0	......	....	....	....
12	2	4, 6	......	10 c.c. S.	5	0	......	....	....	....
13	3	2, 3, 4	......	5 c.c. S.	4	1	(42 days)* 14 days	Typical	....	....
14	1	3 days	......	5 c.c. S.	3	0	......	Moderate	....	....
15	1	2½	......	5 c.c. S.	8	1	16 days	....	3 (1 to 3 years)	2 (12 days) Typ. 1 (13 days) Typ.
16	4	6 m., 9 m., 7 m., 10 m.	......	2.5 c.c. S.	6	0	......			
17	30	1 to 5 yrs.	......	2.5 c.c. S.	6-8	4	16, 17, 17, 20	Moderate	....	....
18	5	1½, 2, 2, 2, 2½	......	3.0 c.c. P.	1-4	0	......	....	....	....
19	4	3, 5, 5, 8	.....	3.0 c.c. P.	1	0	......	Typical	....	....
20	5	4, 5, 8, 8, 9	......	3.0 c.c. P.	4	1	14 days	....	....	....
21	5	2, 3, 3, 3, 7	......	3.0 c.c. P.	4	0	......	....	....	....
22	5	6m., 7 m., 1½, 1½, 2	......	3.0 c.c. P.	5	0	......	....	....	....
23	3	1, 2, 4	......	3.0 c.c. P.	6	0	......	....	....	....
24	3	2, 2, 2½	......	3.0 c.c. P.	6	0	......	....	....	....
Total......	102				...	10	......	7 Mod. 3 Typ.	5	4 Typical

* Forty-two days after the injection of serum and fourteen days after the second exposure.

Group 9.—Two children were convalescing from chicken-pox in one of the wards of the reception hospital, when they were exposed to a case of measles. On the day Koplik's spots were seen in the patient, the third day of exposure, each of the two children received 10 cc. of whole blood and both were protected.

Group 10.—Two adults, who were nurses in training at the Presbyterian Hospital, never had had measles, and were exposed to a colored maid who had developed a measles rash two days previously. Each of the two nurses received 15 cc. of whole blood on the sixth day of exposure, and both were protected.

Group 11.—On the same day the nurses were injected, there was admitted to the emergency ward of the Presbyterian Hospital a little girl with Koplik's spots and a beginning rash. A younger brother, aged 1½ years, was seen at his home the same evening and injected with 10 cc. of whole blood. The child was protected.

The following thirteen groups of children were injected with convalescent measles serum or plasma.

Group 12.—Two children convalescing from scarlet fever were exposed to a child in an adjoining crib, who developed a measles rash the day before. Each child received 10 cc. of convalescent serum on the fifth day of exposure. Both children were protected.

Group 13.—Three children were exposed in the diphtheria pavilion to a child who developed measles fifteen days after admission to the hospital. On the fourth day of exposure, the day the rash appeared, each of the three children was injected with 5 cc. of convalescent serum that had been kept in the icebox for two and one-half months. The children were protected. *One of these children was subsequently exposed to another case of measles twenty-eight days after the serum injection, and following an incubation period of fourteen days he developed a typical attack of measles.*

Group 14.—At the request of the assistant resident physician, the Writer injected a 3-day old colored baby with 5 cc. of convalescent serum. The mother had given birth to the baby on the day the measles rash appeared. The baby remained free from measles.

Groups 15 and 16.—Five children were injected, December 26, in the service of Dr. Schick at Mount Sinai Hospital. They had been exposed to a child who had developed measles two days previously. The four young children, varying in ages from 6 to 10 months, were emaciated feeding cases in which measles would have been a dangerous complication. Each child received 25 cc. of convalescent serum on the sixth day of exposure. Another child, aged 2½ years, was injected two days later (eighth day of exposure) with 5 cc. of serum. The four children injected on the sixth day of exposure were protected. The fifth child, injected on the eighth day, developed a modified mild form of measles sixteen days after exposure. Three other children between 2 and 5 years of age exposed at the same time were not injected. Two of the three children developed typical measles after the usual incubation period.

Group 17.—This group was seen at the Society for the Prevention of Cruelty to Children. It consisted of thirty children under 5 years of age, who were exposed for from six to eight days before receiving convalescent serum. The dose for each child was 2.5 cc. Of the thirty children, four injected on the eighth day of exposure developed a mild, modified form of measles.

Group 18.—These five children were in the diphtheria pavilion and were injected each with 3 cc. of convalescent plasma. They had been admitted to the hospital ward on different days, and were exposed as follows: one child had one day, one had two days and the other three had four days of exposure to a measles patient (Group 13) who had been injected forty-two days previously with convalescent serum. The five children were protected.

Group 19.—Four children were exposed in a small ward at the Ruptured and Crippled Hospital to a child that had been admitted to the hospital the day before. The rash appeared twenty-four hours after admission. Each of the exposed children was injected with 3 cc. of convalescent plasma. No measles developed.

TABLE 4.

SUMMARY OF RESULTS WITH CONVALESCENT WHOLE BLOOD, PLASMA AND SERUM

Days of Exposure to Measles	Total Number Injected	Number That Developed Measles	Period of Incubation Days	Type of Measles
1	5	0	......	....
2	5	0	......	....
3	3	0	......	....
4	26	2	42 14	Typical Typical
5	8	0	......	....
6	17	0	......	....
7	11	1	27	Typical
8	27	7	16-22	Modified
Total	102	10		7 Modified 3 Typical
Controls	5	4	12-15	Typical

Group 20.—Five children in the diphtheria pavilion were injected with 3 cc. of convalescent plasma on the fourth day of exposure. One of the five children developed a fairly typical but moderate rash fourteen days after exposure and ten days after the plasma injection. The elevated temperature persisted for two days. There was some coryza, slight cough and Koplik's spots. The child made a rather rapid recovery.

Groups 21 and 22.—Ten children were exposed in one of the diphtheria wards to two cases of measles. The children in Group 21 received the convalescent plasma on the fourth day of exposure, and those in Group 22 on the fifth day of exposure. No measles developed.

Group 23.—Three children in two families received the convalescent plasma on the sixth day of exposure. The children were protected.

Group 24.—Three children were injected in one of the wards of the pediatric service of Bellevue Hospital. They had been exposed for six days. No measles developed.

Table 5 shows the results with convalescent plasma in two institutions in which measles had developed. It is interesting to note at the Israel Orphan Home the three individual groups of children. Each group was separated from the others, occupied a different floor, and received a dif-

TABLE 5.

EFFECT OF PERIOD OF CONVALESCENCE ON EFFICIENCY OF BLOOD PLASMA IN THE PROPHYLAXIS OF MEASLES

Children Injected											Controls*			
			Convalescent Plasma				Developed Measles					Developed Measles		
Institution	Groups of Children	Date of Injection	No. Days Convalescent	Amount Injected, cc.	Number Children Injected	Age, Years	Period of Incubation. Days	Total	Mild	Typical	Total	Total	Mild	Typical
Israel Orphan Asylum	1st	2/17/24	Mixed 8-15	3	12	1-5	10-12	6	6	0	10	1	0	1
	2d	2/27/24	13	3	20	1-5	10-11	9	9	0	6	2	0	2
	3d	2/29/24	35	3	18	4-7	10-13	7	3	4	17	7	3	4
Total					50	..	....	22	18	4	33	10	3	7
Masters Day Nursery		2/21/24	8	3	8	2-5	17-19	8	5	0	4	3	0	3
Total in both institutions					58	..	....	30	23	4	37	13	3	10

* In some of the controls as well as in some of the injected children at the Israel Orphans Home there was a doubtful history of measles. The parents will at times give a definite history that the child has had measles when the child has really not had the disease, so as to insure its admission into an institution.

ferent preparation of convalescent plasma. In Group 1, a preparation was used consisting of a mixture of three plasmas obtained from the eighth to the fifteenth day of convalescence. Of twelve children injected, six developed a mild form of measles with little or no catarrhal symptoms. One child that was not injected developed typical measles. In Group 2, the plasma of a single donor, thirteen days convalescent, was used. Of twenty injected children, nine developed a mild type of measles; of six children who were not injected, two developed typical measles. In Group 3, a pooled plasma was used obtained from thirty-five convalescent donors. Of eighteen children injected, seven developed measles, three mild and four typical with characteristic catarrhal symptoms. Of seventeen children who were not injected, there were also seven who developed measles, three a mild and four a typical form.

Summary of Cases in Which Injections Were Given

Of the 102 children receiving convalescent whole blood, plasma or serum, ten developed measles. Of these, seven were injected on the eighth day of exposure, and developed a modified form of measles after periods of incubation varying from sixteen to twenty-two days. One child injected on the seventh day of exposure developed typical measles twenty days later, after a period of incubation of twenty-seven days. The tenth child was protected by 5 cc. of convalescent serum for twenty-eight days, when she was exposed to a second case of measles and developed a typical attack fourteen days later, forty-two days after receiving the serum.

Of the five control children, four developed typical measles after periods of incubation varying between twelve and fifteen days.

Of fifty-eight additional children injected in two institutions, twenty-three developed a mild and modified form of measles, and three a typical form of the disease with characteristic catarrhal symptoms. In these three children, a thirty-five day convalescent plasma was used in doses of 3 cc. Of thirty-three control children who were not injected, three showed a mild form and ten a typical form of measles after the usual period of incubation.

For bibliography see the original article.

SUSCEPTIBILITY OF RABBITS TO THE VIRUS OF MEASLES

Inoculations with Nasopharyngeal Material

Published in The Journal of Infectious Diseases, 1922, Vol. XXX, pp. 86-90

M. Grund

The investigation, the results of which are reported now, was undertaken to determine whether the inoculation of nasal secretions from patients with measles would produce definite and characteristic symptoms in rabbits; it was carried on at the same time, and with material from the same patients as were used by Nevin[1] in her work on blood inoculation.

The nasopharynx of patients in the early eruptive, or pre-eruptive stage of measles was irrigated with 30 to 50 cc. normal salt solution. Cultures were made, from the material thus obtained, on blood-vitamine-agar, to establish the prevalent types of bacteria. From 5 to 10 cc. of the washings were injected into the trachea of rabbits, the animals being lightly anesthetized. In most animals this amount of fluid caused a leakage from the nose, showing that the mucous membrane of the upper air passage was thoroughly flooded with infectious material. Unfiltered washings were used in most cases, as it was believed that the usual flora of the upper respiratory tract would not cause characteristic symptoms in rabbits. Only a small number of rabbits received material passed through a Berkefeld V candle. Aerobic cultures from these filtrates were uniformly negative for the test organism (B. prodigiosus).

The majority of rabbits (table 1) gave a certain reaction, yet apparently there were among them a fairly large number of refractory individuals for, in a number of instances in this series, only one animal out of two inoculated with the same nasal washings developed symptoms, and even when both succumbed there was often considerable difference in the severity of symptoms. In susceptible animals the incubation period varied between two and seven days.

The least reliable and constant symptom seems to be the enanthem; while present in about 20% of the cases, only 5 animals showed what one might describe as good typical Koplik spots. The temperature curve also is not at all characteristic; in very few animals did the temperature go above 103°F. In some of the cases a certain relationship appeared between the temperature curve, the cutaneous symptoms and the leukocyte count; however, it was far from constant, and the fluctuations noted in daily blood counts made for one week before inoculation do not make a "leukopenia" seem a very dependable diagnostic sign in rabbits. In a number of animals a distinct polychromatophilia was noted.

Conjunctivitis and inflammation of the upper respiratory passages, in varying degrees of severity, occurred in 70% of animals; it is not always possible to eliminate "snuffles," but the appearance of coryza together with the rash, and its brief duration in most cases, point against its being an intercurrent infection. Seventy-five per cent. of the rabbits showed some form of cutaneous eruption, either a diffuse, punctuate erythema, which in 3 severe (2 fatal) cases became petechial, or, sometimes following the

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[1] J. Infect. Dis., 1921, 29, p. 429.

TABLE 1

RABBIT INOCULATIONS WITH NASOPHARYNGEAL WASHINGS

Case	Rabbit No.	Exanthem						Desquamation			Coryza			Conjunctivitis			Enanthem		Temperature Extremes	Leukopenia			Remarks
		Erythematous			Maculopapular																		
		Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset		Intensity	Day of Onset	Duration	
M19	191	+	2	2	..	..	..	..	..	..	+	9	2	..	..	..	..	..	102.2-102.8	..	..	..	
	192	+	2	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	102 -102	..	..	..	
M20	201	..	..	..	..	..	..	..	..	..	+	12	1	..	..	..	..	..	102.6-102.8	+	10	1	Died after 4 days; dysentery
M21	211	..	..	..	..	..	..	..	..	..	+	3	4	+	3	1	+?	6	101.3-103	+	2	1	
	212	++	2	2	..	..	..	..	..	..	+	2	2	+	2	1	..	..	102 -105	++	3	1	
M22	221	+	2	4	+	4	2	+	5	2	+	4	2	+	4	1	..	..	100.8-102.4	+	3	1	Died after 4 days; bronchopneumonia
	222	..	..	..	..	..	..	+	6	3	..	..	..	..	..	..	..	..	99.8-102.2	+	2	2	
M23	231	+	2	4	..	..	..	+	5	6	+	3	1	..	..	..	..	..	102 -104.8	..	..	..	Filtered material inoculated
	232	++	2	5	++	4	3	++	4	7	+	5	3	..	..	..	..	..	100.8-102	+	3	1	
M24	241	+	3	3	..	..	..	+	6	5	..	..	..	..	..	..	..	..	99.4-100.8	..	..	..	
	242	..	..	..	..	..	..	+	7	5	..	..	..	..	..	..	..	..	99.6-103.1	+	3	1	
M25	251	..	..	..	+	7	2	+	10	3	+	5	6	+	10	1	..	..	101.8-104.7	+	7	1	
M26	261	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	101.7-105.4	+	4	1	Phlegmon of abdominal wall
	262	..	..	..	..	..	..	+	10	3	+	7	1	..	..	..	..	..	102. -105.8	+	5	1	Died of rabbit septicemia
	263	+	2	7	..	..	..	+	11	5	..	..	..	..	..	..	..	..	101.8-107	±	2	1	
M27	271	++	2	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	101.4-101.3	++	2	2	
M29	291	..	..	..	±	3	2	±	12	4	±	2	6	..	..	..	+	2	....	..	..	..	Died after 4 days; broocho-pneumonia
	292	±	2	2	..	..	..	+	14	5	++	3	4	..	..	..	..	..	....	..	..	..	Filtered material innoculated
R f	R 1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	....	..	..	..	
D 1	D 1	..	..	..	+*	2	..	+*	5	..	+	2	..	+	2	..	..	..	....	..	..	..	Died within 24 hours
D 11	D2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	....	..	..	..	
Sc I	S1	..	..	..	..	..	..	+	1	..	..	..	..	..	..	..	..	..	....	..	..	..	
Sc II	S2	+	4	..	..	..	..	+	7	..	+	3	..	..	..	..	..	..	....	..	..	..	

*Scratches ?

TABLE 2

PASSAGE EXPERIMENTS AND INOCULATIONS WITH CULTIVATED VIRUS

Case	Rabbit No.	Exanthem						Desquamation			Coryza			Conjunctivitis			Enanthem		Temperature Extremes	Leukopenia			Remarks
		Erythematous			Maculopapular																		
		Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset	Duration	Intensity	Day of Onset		Intensity	Day of Onset	Duration	
M21	213	+	2	2	..	..	..	±	4	1	+	2	2	+	2	1	±?	2	102.2-102.4	+	3	1	Inoculated with filtered lung tissue from R 212
	214	±	3	1	..	..	..	..	..	..	+	3	1	..	..	..	..	..	101.6-102.2	..	..	..	Inoculated with filtered blood culture R 212
	215	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	101.6-102	..	..	..	Inoculated with nasal discharge from R 212
M27	272	..	..	..	+	5	1	+	6	2	+	5	2	..	..	..	..	..	102.3-103.6	++	7	1	Inoculated with unfiltered lung tissue R 271; died 8th day
M28	281	++	2	5	..	..	..	+	5	4	+	2	2	±	2	2	±	1	103 -103	..	..	..	Both inoculated with 2d transfer of a culture of filtered nasal washings, M 28
	282	+	2	2	+	2	2	+	7	7	±	2	2	..	..	..	+	2	102 -102.4	..	..	..	
	283	+	3	2	+	3	2	+	13	6	+	5	1	..	..	..	+	2	....	..	..	..	Inoculated with blood from R 281-282 (pooled)
	284	..	..	..	..	..	..	..	..	..	+	3	4	..	..	..	+?	3	....	..	..	..	Inoculated with blood from R 283
	285	±	3	1	+	5	5	+	10	6	++	4	6	..	..	..	..	..	....	..	..	..	Both inoculated with blood culture from R 281, second transfer
	286	+	3	4	..	..	..	+	10	5	+	3	4	..	..	..	..	..	....	..	..	..	
M29	293	..	..	..	+	6	3	+	11	3	+	2	6	..	..	..	..	..	....	..	..	..	Inoculated with culture from filtered nasal washings; patient M 29, second transfer
	294	++	2	8	..	..	..	+	9	5	++	2	4	..	..	..	++	2	102	..	..	..	
	295	..	..	..	+	4	2	..	..	..	..	..	..	..	..	..	..	..	....	..	..	..	Inoculated wtih culture from filtered nasal washings, patient M 29, third transfer
	296	+	2	1	+	3	2	..	..	..	+	7	2	+	2	1	+	2	....	..	..	..	
	297	+	4	2	+	5	4	+	10	3	+	5	2	..	..	..	..	..	....	..	..	..	Inoculated with culture from filtered nasal washings; M 29, fifth transfer

erythema, sometimes occurring without it, a maculopapular rash, which faded after 2 to 4 days and left pigmentation persisting until desquamation began.

Desquamation, either branny or flaky, occurred in all but 4 animals after the rash and was noted 3 times in cases in which no rash had been noted (3 animals with marked erythema died before desquamation occurred).

Control animals were inoculated with nasopharyngeal washings from 1 case of nasal catarrh, 2 cases of diphtheria and 2 of scarlet fever. There was desquamation in the 2 scarlet fever animals, preceded in one case by a rash which was, however, different in character from that shown by the measles rabbits. The other animal, showing 2 macules, presented other evidences of clawing and scratching over some of the unshaved portions of his skin.

Passage experiments from rabbit to rabbit were unsuccessful when nasal discharges were used, owing probably to the scantiness of material. Successful inoculations were made in 7 cases, using, 3 times, 2 cc. of blood, and, twice, a suspension of lung tissue from severe and fatal cases. The other passage experiments were conducted with rabbit blood cultures. Attempts have also been made to cultivate a specific organism from the filtered nasal washings from patients, and while the work along these lines is incomplete, the reactions obtained in rabbits inoculated with the fifth transfer of such cultures indicated at least that the virus remains alive and virulent at 37°C. for 24 days. The results obtained by reinoculating convalescent animals have thus far been rather contradictory, and frankly successful in only two cases.

It cannot be claimed that the results obtained in any one animal give a clear and typical picture of measles, yet, taking the series as a whole, there has been enough conformity to encourage the belief that rabbits are susceptible to the virus of measles and within rather wide limits give a characteristic syndrome.

Note.—E. Harde, in the Compt. Rend. Soc. de biol., 1921, 84, p. 968, makes a short communication on the transmission of measles from man to rabbit by means of intravenous inoculation with blood. She states that an erythema develops within 48 hours and lasts from 24 to 48 hours. The blood of a scarlet fever patient produced only a slight reddening after the same interval of time, but no real erythema. These injections were made at this laboratory a number of years ago, but the results were not considered conclusive enough to follow up or to publish, until Dr. Harde saw the rabbits described in the present articles.

THE PROPHYLACTIC USE OF MEASLES CONVALESCENT SERUM

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William H. Park and Rowland G. Freeman, Jr.

As measles is the most prevalent of the diseases of infancy and early childhood, and also predisposes to a complicating bronchial pneumonia, it is one of the two most fatal of the communicable diseases of early childhood, and its prevention has been eagerly sought by clinical and laboratory investigators. The ideal in their mind is to discover the germ and prepare a vaccine such as we have against diphtheria, tetanus and scarlet fever. Tunnicliff in the United States and Caronia in Italy have reported such discoveries. Their work is now being subjected to crucial tests.

TABLE 1.

TOTAL DEATHS AND DEATHS FROM MEASLES

	1918	1919	1920	1921	1922
Total deaths	98,119	74,433	73,249	64,257	69,690
Total deaths under 5	21,019	15,744	17,288	13,672	14,847
Total deaths from measles	790	218	736	165	977
Deaths from measles under 5	734	210	687	150	917
Percentage under 5	4	1	3.5	1	6

Some thirty years ago, a German physician used large amounts of serum from convalescents in the treatment of several cases in which the symptoms were just beginning. The results were favorable, and he suggested that it might be used as a preventive measure.

Since 1921 the convalescent serum has been utilized with excellent results in the hospitals for contagious diseases in New York City, in children who have been exposed by the development of measles in the wards. A grant of money by the Metropolitan Life Insurance Company made it possible to attempt to provide enough serum for all exposed inmates in children's institutions in New York City, and also for all the children in the city who were under three years of age.

Results Obtained in New York City

More than 1,500 children received preventive injections between December 1 and June 1. From 979 of these we have fairly accurate data as to the date and degree of exposure, the amount of plasma injected and the success attained.

Of these 979 children, 753 were exposed in institutions and 226 in private families. In 404 of the institutional children and in all of the children in families, the exposure was direct. In 349 it was indirect, in that actual personal contact with a measles patient was not proved.

No bad effect followed the injections in any case. Only one of the children died, and this one had an empyema before contracting modified measles and was only 2 years of age. We do not think that the attack of measles appreciably hastened its death.

TABLE 2.
DATA IN INSTITUTIONAL CHILDREN RECEIVING PREVENTIVE INJECTIONS

Amount of Plasma Injected	Number of Children	Modified Measles, Per Cent.	Unmodified Measles, Per Cent.	Complete Success, Per Cent.	Incomplete Success, Per Cent.
3 cc.	219	20	8	72	92
4 and 5 cc.	190	8	2	90	98
6 to 10 cc.	243	6	2	92	98
Total in institutions.....	753	11	5	84	95
Total in families........	226	42	5	52	95

CONCLUSIONS

The injection of 6 cc. of convalescent serum or plasma into a child under 3 years of age and from 6 to 10 cc. for a child over that age who has been exposed for less than five days to measles, is sufficient, as a rule, to prevent infection. If it does not, it almost certainly so modifies the attack that it is very mild and is not likely to lead to complications.

The prompt use of the serum will prevent serious outbreaks of measles from developing in institutions and so prevent the development of complicating pneumonias.

It would be of extreme value to have a supply on hand to prevent outbreaks of measles among troops, such as occurred so disastrously in the late war.

The serum gives immunity for only two weeks to one month. In the modified cases there is lasting immunity.

The serum gives the greatest accumulation of antibody shortly after the convalescence of the patient. At the end of three months, the antibodies are still abundant but probably less in amount.

In order that the use of convalescent measles plasma may become general, some plan must be devised by which the supply will be constant. It would seem that if every physician equipped himself with the very simple apparatus necessary for obtaining the blood, and if the attempt was made to draw between 10 and 25 cc. of blood from every case seen in children, and larger amounts in adults, he would have enough for the majority of exposures in his practice, the average dose being only 5 cc. Institutional needs must be met by other methods, such as the one to which the New York City Department of Health had recourse. The Department of Health has a standing offer to send a physician to any convalescent adult who has given consent to have his blood drawn. After the blood is withdrawn a fair proportion is kept for the use of the physician who referred the case to·the department.

THE RELATION OF THE TUNNICLIFF AND FERRY DIPLOCOCCI TO MEASLES

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William H. Park, Anna W. Williams and Minnie Wilson

This paper is entirely concerned with the study of the diplococcus which has been suggested as the cause of measles by Dr. Tunnicliff and of similar green producing streptococci claimed to be the cause of measles by Dr. Ferry.

As is well known, Tunnicliff in 1917 described a micrococcus which she obtained from the blood of early cases of measles. She did not obtain this regularly from the cases at first, giving as her reason that they grew with difficulty, but later she almost never failed to obtain them. There were certain cultural differences in the strains. For instance, one strain was definitely an anaerobe and some did and some did not produce acidity in milk. She believes that with this culture she successfully produced measles in rabbits and noted that these rabbits after their recovery became resistant to later inoculations. These effects were produced not only with the primary cultures but with those of the fifth transfer. She observed that strains of other types of streptococci obtained from the blood either early or late in the disease did not produce measles. This seemed to her to make unlikely the claim that it was not the diplococci but rather a filterable virus which had grown in symbiosis with the supposedly specific green diplococci, which produced the disease. She found that while a filtrate alone or the dead diplococci alone failed to give any distinctive diagnostic cutaneous reaction, that the whole culture fluid containing both cultures and toxic fluid did give differential results between immune and non-immune children and animals. The serum of immunized animals also neutralized the toxic culture fluid. She stated that the mixed culture fluid and organisms regularly produced a reaction in the skin of children who had not had measles, but not in convalescent measles patients or in 96 per cent. of those who gave a history of having had measles. She further stated that the serum of goats injected with the cultures, when injected into children within two days of exposure to measles, prevented the disease in the majority and modified it in the others a little later.

Ferry obtained from the blood of an early case of measles a green streptococcus which made a strong toxin. In his hands 0.1 cc. of a 1 to 400 dilution of the filtered toxin produced a skin reaction in the great majority of susceptible children and very rarely in those who had had measles. He produced in animals an antitoxin for the toxin. He considered his culture as distinct from the ones isolated by Tunnicliff, because of her statement that hers was a diplococcus and that it did not make a soluble toxin. In our hands both cultures make a toxin, but that from Ferry's culture is much stronger. They seem to us to be similar organisms.

The published reports of Tunnicliff and of Ferry certainly seem to present very impressive evidence of the etiological significance of a diplococcus growing in short chains. There were, however, several difficulties which prevented us from being completely convinced. It seemed difficult to understand how a diplococcus which appeared in enormous numbers in

TABLE 1.

SHOWING PERCENTAGE OF CASES FROM WHICH GREEN STREPTOCOCCI (NON-HEMOLYTIC) WERE ISOLATED AT VARIOUS STAGES OF MEASLES

			Green Streptococci		
			Positive	Doubtful	Negative
Stage of Disease	No. of Cases	Percentage of Cases from which Green Colonies Were Isolated	Green and Ascitic Colonies Like Tun. No. of Case	Green Col. Like Tun. Ascitic Col. Not Like Tun. No. of Cases	Col. Not Green Morphology of Col. Like Tun. Ascitic Col. Not Like Tun. No. of Cases
Pre-eruptive	20	70	13	2	5
First day rash	19	89	16	1	2
Second day rash	11	72	7	1	3
Old rash	7	71	5	0	2
Controls:					
So-called influenza	9			9	

The cultures in our "Positive" column correspond to the description given by Tunnicliff of the colonies isolated from rubeola. The following is her description: On blood agar the colonies are small punctate, green. On ascitic agar, the colonies are small, round, homogeneous, smooth-edged.

the discharges from the eyes, nose and throat during the catarrhal stage of measles should be absent in cases, as shown by its lack of contagiousness, from the upper respiratory tract during the period of incubation. If measles were due to this diplococcus it would seem probable that, like diphtheria and scarlet fever, soon after the transfer of the infecting microbe it would begin to develop and produce symptoms and spread infection.

Again in almost all diseases due to bacterial infection there are a greater or lesser number of cases in which the infecting microbes persist. Cultures as well as transmitted infection to others reveal this. Measles, like smallpox, mumps, chickenpox, yellow fever and most other diseases supposed to be due to filterable viruses, has a long period of incubation when it is not communicable and then, when convalescence is established, it suddenly ceases to spread contagion.

While impressed by the evidence submitted by Tunnicliff and Ferry we were still doubtful and we felt it wise to undertake seriously an attempt to corroborate their findings. In order to make these studies possible we applied for and obtained a grant of money from the Milbank Memorial Fund. To our force we added a number of technical assistants.

Our first endeavor was to discover through cultures whether the diplococci were regularly present in the pre-eruptive and eruptive stages and whether the strains were the same in the different cases. It is only fair to state that the cultures tested by us were from the mucous membranes and not from the blood, but many of them were identical with either the Tunnicliff strain or the Ferry strain. The results of our work are shown in the tables presented here.

Table I gives the results so far as the appearance of colonies on blood and ascitic agar indicate identity.

The results viewed from the etiological standpoint are perplexing. Eighty-four per cent. of the cultures from the measles cases gave characteristic colonies on both blood and ascitic agar, while in cultures from 9 control cases characteristic colonies were not found. They were absent, however, in the cultures from 16 per cent. of the measles cases and their persistence during the period of the fading rash was somewhat disquieting since the cases had reached a stage when some were supposed to be but slightly and others not at all communicable.

Twenty-two of these strains (see Table 2), together with the Tunnicliff and Ferry strains sent us by these investigators were studied as to their action on carbohydrates. Six different groups were found, only two of which contained many cultures, namely, the Tunnicliff and our 67 T. It is striking that the Ferry and Tunnicliff strains are different as to their action on salicin and inulin. These findings are somewhat disconcerting and rather suggested the possibility that these diplococci are complicating micro-organisms. We then tested different strains for their reaction to the agglutinin developed by the Tunnicliff strain, the Ferry strain and our 67 T strain. (See Table 3.)

TABLE 2.

22 STRAINS TYPED BY CARBOHYDRATES AS COMPARED WITH TUNNICLIFF'S AND FERRY'S

						No. of Our Strains in This Type	
Type	Maltose	Salicin	Inulin	Mannite	Raffinose	No.	Stage of Disease
Tunnicliff	+	+	+	—	+	6	2 pre-eruptive 3 1st day rash 1 8th day convalescence
Our 2. E..........	+	—	+	—	—	2	1 pre-eruptive. This is tonsil type of culture case 1 3rd day of rash
5. T..........	+	—	+	—	+	2	4th day of convalescence
4. T..........	+	+	+	—	—	0	
67. T..........	+	+	—	—	+	7	1 pre-eruptive 1 1st day of rash 5 2nd day of rash
Ferry	+	—	—	—	+	1	11th day of convalescence

The strains shown in Table II are similar to Tunnicliff's on blood-agar, ascitic-agar and in bile test.

TABLE 3.

COMPARISON BY DIRECT AGGLUTINATION OF OUR GREEN STREPTOCOCCI WITH TUNNICLIFF'S AND FERRY'S

Cultures Isolated from Various Stages of Measles

			Agglutinin Serums					
			Tunnicliff		Ferry 01860		Our 67-F	
Stage of Disease	Bacterial Suspensions Strain Number	Rabbit Serum No.	Titre with Homologous Antigen	Titre with Other Strains	Titre with Homologous Antigen	Titre with Other Strains	Titre with Homologous Antigen	Titre with Other Strains
	Tunnicliff ...	20	3200		1600	—	3200	3200
	Ferry 1		3200	—	1600		3200	—
	Ferry 2		3200	—	1600	1600	3200	—
Pre-eruptive	67-T..........	31	3200	3200	1600	—	3200	
	2-E..........	20	3200	—	1600	—		
	T..........		3200	—	1600	—		
	4-T..........	C	800	—	1600	1600		
	9-E..........		800	—	1600	—		
	27-E..........	20	3200	100	1600	—	3200	—
	33-T..........		3200	—	1600	—	3200	—
	91-E.	20	3200	—	1600	—	3200	—
First Day Rash	1-E..........	20	3200	—	1600	—	3200	—
	N..........		3200	—	1600	—	3200	—
	18-E..........	C	800	—	1600	1600		

— Means no agglutination in 1:100 dilution.

Explanation of Strain Number: The numeral refers to serial number of the case, the letter to source of the culture: E (eye), N (nose), T (tonsil)

It is noted that the rabbit blood serum which had been removed from the rabbit treated by injections of the Tunnicliff strain agglutinated strongly only one of the strains other than the Tunnicliff and only one other in very low dilution. The serum from the Ferry rabbit serum agglutinated 2 of the 12 strains, and the serum from our 67 T none of the 7 strains tested. We then tested the strains by agglutinin absorption and found the same results as by direct agglutination. (See Table 4.)

TABLE 4.

OUR GREEN STREPTOCOCCI FROM MEASLES COMPARED WITH TUNNICLIFF'S AND FERRY'S BY AGGLUTININ ABSORPTION

		Agglutinin Serums				
		Tunnicliff			Ferry 01860	
Stage of Disease	Absorhing Cells Strain No.	Rabbit Serum No.	Titre with Homologous Antigen Before Absorption with Cells Listed Under "Strain Number"	Titre with Homologous Antigen After Absorption	Titre with Homologous Antigen Before Absorption	Titre witb Homologous Antigen After Absorption
	Tunnicliff	20	3200	—	1600	1600
	Ferry 1..		3200	3200	1600	—
	Ferry 2..		3200	3200	1600	—
Pre-eruptive	67-T.....	20	3200	—	1600	800
	2-E.....		3200	3200	1600	1600
	T:....		3200	3200	1600	400
	4-T.....	C	800	800	1600	1600
	9-E.....		800	800	1600	800
	27-E.....	20	3200	1600	1600	800
	33-T.....		3200	3200	1600	400
	91-E.....		3200	3200	1600	800
First Day Rash	1-E.....		3200	3200	1600	1600
	N.....		3200	3200	1600	1600
	18-E.....	C	800	800	1600	200

— Means no agglutination in 1:100 dilution.

Explanation of Strain Number: The numeral refers to serial number of tbe case, tbe letteer to source of the culture: E (eye), N (nose), T (tonsil).

These results are not incompatible with the possibility that, while these strains differ so markedly in their reaction to specific agglutinins, they may produce the same toxin. We know that there are many different agglutinative types of diphtheria bacilli, scarlet fever streptococci and pneumococci, but the great diversity of these strains suggests that they, like the influenza bacilli which have the same great diversity, may be only complicating slightly pathogenic bacteria.

When the work was begun we planned to make cultures from a few of the cases from time to time from the beginning of the disease until after convalescence, but in the mass of work the making of the later cultures was put off until June, when the cases suddenly ceased. We had intended to test the early and later cultures from the same case for their identity in relation to specific agglutinins. This work is now being undertaken but it was impossible to complete any of the cases in time to make a report at this time.[1] We can state, however, that in 3 cases of measles the green diplococci persisted up to the tenth, twenty-first and twenty-third day after the appearance of the rash and that, so far as cultural appearance could determine, the diplococci which appeared in the first cultures made early in the disease persisted to the final cultures made during convalescence and after. (See Table 5).

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[1] October 13, 1926.

TABLE 5.

SHOWING PERCENTAGE OF GREEN STREPTOCOCCI IN CULTURES FROM CONVALESCENT MEASLES

		Eye		Nose		Tonsil	
Case Number	Days Convalescent	Amount Growth	Per cent. Green	Amount Growth	Per cent. Green	Amount Growth	Per cent. Green
98	6	+	100	+++	0.1	+	100
	8	+	100	+	100	No. growth	
	21	+	90	+	75	+++	95
99	11	++	95	++	90	++	99
	16	No growth		±	95	±	99
	23	+	45	+	25	++	50
100	6					+	50
	14					++	50
101	3					±	100
	7					+	50
	10					±	100

± = Scant growth. + = Moderate growth. ++ = Abundant. +++ = Profuse.

If further investigation proves them to be identical it would require conclusive proof to make us accept them as the cause of measles, for it is well established that cases of measles are not communicable at such a period after convalescence and we have no knowledge of such a rapid loss of virulence in streptococci in convalescence.

Drs. Tunnicliff and Ferry both claim that, when the toxin and dead organisms are used in proper dilution in intracutaneous tests, they are diagnostic between immunity and lack of immunity to measles.

We have observed the results of tests made in hundreds of young children, some of whom have had measules and some of whom have not,

TABLE 6.

TESTS MADE WITH TUNNICLIFF MEASLES TOXIN, WILLARD PARKER

Case	Age	Toxin 1:20 3	Dilutions 1:40 3	Toxin 1:10 aa Convalescent Serum 1:2	Broth Control 1:20
		"Suscept bles"—i e., those who never had measles			
1	4 years	1 x 1 P	1 x 7 PP	.8 x .8 P	
2	16 months	.4 x .4 P	.4 .4 P	1.5 x 1.5 P	.5 x .4 P
3	2 years	.4 x .3 P	.3 x .3 P	.5 x .5 P	.2 x .2 P
4	15 months	1.5 x 1.5 P	1 x 1 P	.6 x .* P	.5 x .5 P
6	14 months	.5 x .4 P	.7 x .6 P	.5 x .6 P	.3 x .4 P
6	4 years	1.5 x 1.5 P	2 x 2 P	.5 x .5 P	
7	6 years	.5 x .6 PP	.3 x .3 PP	.9 x .9 P	
	"Non-susceptibles"—i.e., the following children give a definite measles history				
1	3 years	1.2 x 1.1 P	.9 x 1.1 P	2.5 x 2.5 BP	
3	2½ years	.7 x .8 P	.5 x .* P	.6	
2	2½ years	1.2 x 1.3 P	.7 x .8 VPP	1.5 x 1.7 PP	.5 x .4 P
4	2 years	.5 x .5 P	.4 x .4 P		.2 P
5	1 year	1.2 x 1.2 P	1.5 x 1.6 P	1.5 x 1.4 P	
6	3 years	.2 x .2 PP	.2 x .2 PP	.6 x .7 P	
7	4 years	1.5 x 1.5 P	.4 x .5 PP	1 x 1.2 BP	

The tests were read at the end of 18 hours.
P = Pink. BP = Bright Pink. PP = Pale Pink. VPP = Very Pale Pink.
1.2 x 1.6 = 1.2 centimeters by 1.5 centimeters.

and we have been unable to obtain distinctive results. Dr. Schroder and Dr. Zingher have obtained very similar results in the children tested by them.

Three years ago with Freeman and Valentine we tried to see if it were possible to get in some medium the development of some toxin in cultures from filtered nasal secretion of early cases of measles. In only one case did we get a toxin, and this curiously enough was in a culture in which a diplococcus like the Tunnicliff diplococcus had passed the filter. This gave us fairly distinctive differences between susceptible children and those who had recently recovered from measles. Those who had not had it recently reacted quite irregularly.

It seemed to us, therefore, that there might have been some reaction to the toxin of the diplococci in the blood and in the respiratory passages but that this in no way proved that they were the original etiological factor. Table 6 gives a characteristic test. It is seen how irregular the reactions were. Certainly they would not suffice to test the immunity of the children. There was practically no difference between the two groups. Similar results were obtained with the Ferry toxin.

EFFECT OF INTRACUTANEOUS INJECTIONS OF THE SERUM OF IMMUNIZED GOATS

Tunnicliff describes the result of injecting her diluted immunized goat serum intracutaneously into children before the measles rash has developed and notes that, just as has been shown to occur with convalescent measles serum, the injection of serum from goats which have received injections of her diplococcus antigen inhibits the development of the rash. Long before her paper appeared we had tried her goat serum, the Ferry serum and serum from goats immunized with our strains intracutaneously in children. Our strains were from cases in the Koplik spot period. The results with all these serums were striking and for a time we felt that we had strong evidence as to the specificity of the diplococci. In scarlet fever, in which intracutaneous injections of convalescent human serum or of antitoxin developed in the horse blanches the developed rash, we think the effect is due to a direct antitoxic action. In preventing the development of the measles rash we are less certain that this is a specific antitoxic reaction. Having obtained these striking results, we tried as controls the serum of a goat which had been immunized to scarlatinal streptococci and one which had received

TABLE 7.

MEASLES EXTINCTION TESTS

Number of Case	Human Measles Convalescent Serum	Goat Serum Our Strains	Goat Serum Tunnicliff Strain	Goat Serum Ferry Strains	Control Animal Serum	Control Animal Serum
	1:10	1:10	1:10	1:10	1:10	1:10
5	2. x 2. F	2. x 2. M			3. x 3.5	1.5 x 1.5 F
6	4. x 5.	4. x 3.			3. x 4.	
9	4. x 5.	3. x 3.	2. x 3.	4. x 3.		
4	3. x 3. M	3. x 3. M	3. x 3.			
9	4. x 5. M	3. x 3.	2. x 3.	4. x 3.		
12	4. x4.	3. x 3.		1. x 1.		

F = Faint blancbing. M = Marked blanching.

diphtheria bacilli and one which had received, so far as known, no injections. The results were surprising. The serum from one of the control goats, when given intracutaneously, inhibited the rash almost as well as did the convalescent measles serum or the Tunnicliff serum and two of the others had definite but not so decided action. Some of these tests are shown in Table 7.

These surprising results throw doubt on the value of the measles rash extinction tests as indicating a specific neutralization of toxin.

We close our paper at this point since we have had no experience with cultures reported upon by Caronia or Degkwitz. The results obtained by Degkwitz have impressed us very strongly and we hope to try and corroborate his findings in the near future.

For bibliography see the original article.

EXPERIMENTAL MEASLES IN RABBITS AND MONKEYS

Published in The Journal of Infectious Diseases, 1921, Vol. XXIX, pp. 429-436

Mary Nevin and Florence R. Bittman.

In our search of the literature on experimental measles we have been unable to find any mention of attempts having been made to transmit the virus to rabbits. Anderson and Goldberger speak of the apparent insusceptibility of animals other than the monkey to the inoculation of measles virus, but no reference is given.

In order to test the receptivity of rabbits to the infection of measles, the blood of 6 patients with measles was inoculated into 6 rabbits. Three of the rabbits were inoculated with blood drawn on the second day, 2 rabbits with blood drawn on the third and 1 with blood drawn on the fourth day after the onset of the disease. In all we inoculated 17 rabbits, 6 with human blood and 11 with the blood of rabbits that had given evidence of a reaction. The inoculations were given intravenously in amounts varying from 1 to 15 cc. All of the rabbits inoculated with human blood gave evidence of a reaction. So also did all but of the 2 rabbits subinoculated from these 6 rabbits. Passage inoculations for subsequent injections into monkeys were carried out only from patient "S."

Symptoms in Rabbits

Fifteen rabbits developed symptoms in from 3 to 7 days. The symptoms were not so marked as in monkeys. In some instances there was a rise in temperature coincident with a decrease in the total leukocyte count, but this was by no means constant. We noted also that in the majority of the rabbits there was frequently a leukocytosis on the second, third or fourth day after injection followed by a decrease in the total leukocyte count. The fact that these findings were not constant, together with the fact that we had found great fluctuations in the daily counts of these animals for one week prior to their inoculation, led us to disregard these changes in the white blood counts.

Of the 15 rabbits showing symptoms of measles, 10 developed small hyperemic, slightly elevated spots on the labial mucosa; in 5 instances these spots showed whitish centers. Twelve rabbits developed, in from 2 to 4 days, a marked conjunctivitis with a moderate edema of the lids and lacrimation. Four rabbits developed a slight diarrhea.

In from 3 to 8 days all of the 15 animals showed erythema of varying degrees, from a slight flush over the chest and in the axillary region to a general redness extending to the groin. A redness of the skin always followed the shaving of the rabbits, but, unlike the control animals noted in the following, the flush became more intense in from 3 to 8 days.

In no instance was a distinctly typical exanthem noted. In 8 rabbits we noted several erythematous macules on the chest and abdomen. Such a rash was not distinctive. Neither is the reaction on the skin of rabbits after inoculation with cowpox vaccine the typical one that takes place in human beings.

In all of the 15 rabbits a marked desquamation occurred, beginning from the fifth to the fourteenth day. Both the shaved and unshaved portions of the neck, chest, axillae, abdomen and groin were involved. In two instances the desquamation was fine and branny; with the remaining 13 animals large flake-like scaling took place.

From one of the human cases, "S," the virus was passed through 5 rabbits (Charts 1, 2 and 3). On the sixth day after inoculation, the fifth rabbit was bled from the heart and the citrated blood injected intravenously into monkey 8, M. rhesus. The monkey developed a leukopenia in 3 days (Chart 4A). On the fourth day there was loss of appetite, slight reddening of the eyelids and lacrimation with a marked erythema over the face, neck and shoulders, while on the labial mucosa two discrete, elevated, hyperemic macules with bluish white centers appeared. On the fifth day a maculopapular rash appeared about the mouth, cheeks and forehead. On the sixth day, over the bluish abdomen of the monkey, small clusters of yellowish, elevated papules appeared. From the sixth to the ninth day there was a moderate erythematous, granular rash on the mucous membrane of the lips. The papules on the labial mucosa disappeared on the eighth day. The rash also began to fade and marked desquamation was noted on the face, chest, shoulders, abdomen and in the groin.

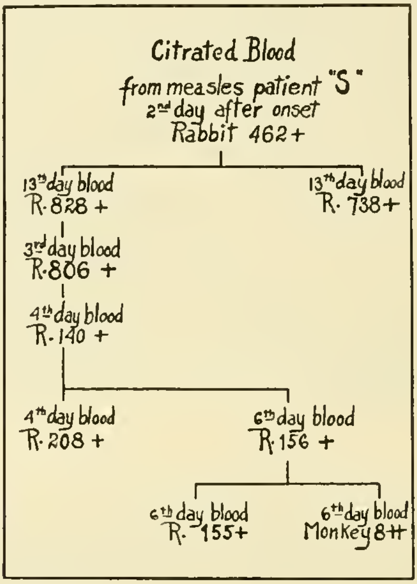


Chart 1.—Transmission of measles virus from case "S" through 5 rabbits to a monkey; suggestive reaction +, typical measles reaction ++.

(Reprinted by courtesy of The Journal of Infectious Diseases.)

Blake and Trask have shown that monkeys inoculated intratracheally with nasopharyngeal washings from patients with measles develop characteristic symptoms of measles. Accordingly, we reinoculated monkey 8, M. rhesus, one month later with nasopharyngeal washings containing another strains of measles virus (case "O"). The monkey failed to react (Chart 4B). The control (monkey 24, M. rhesus, chart 5A) developed the characteristic symptoms of measles in 6 days.

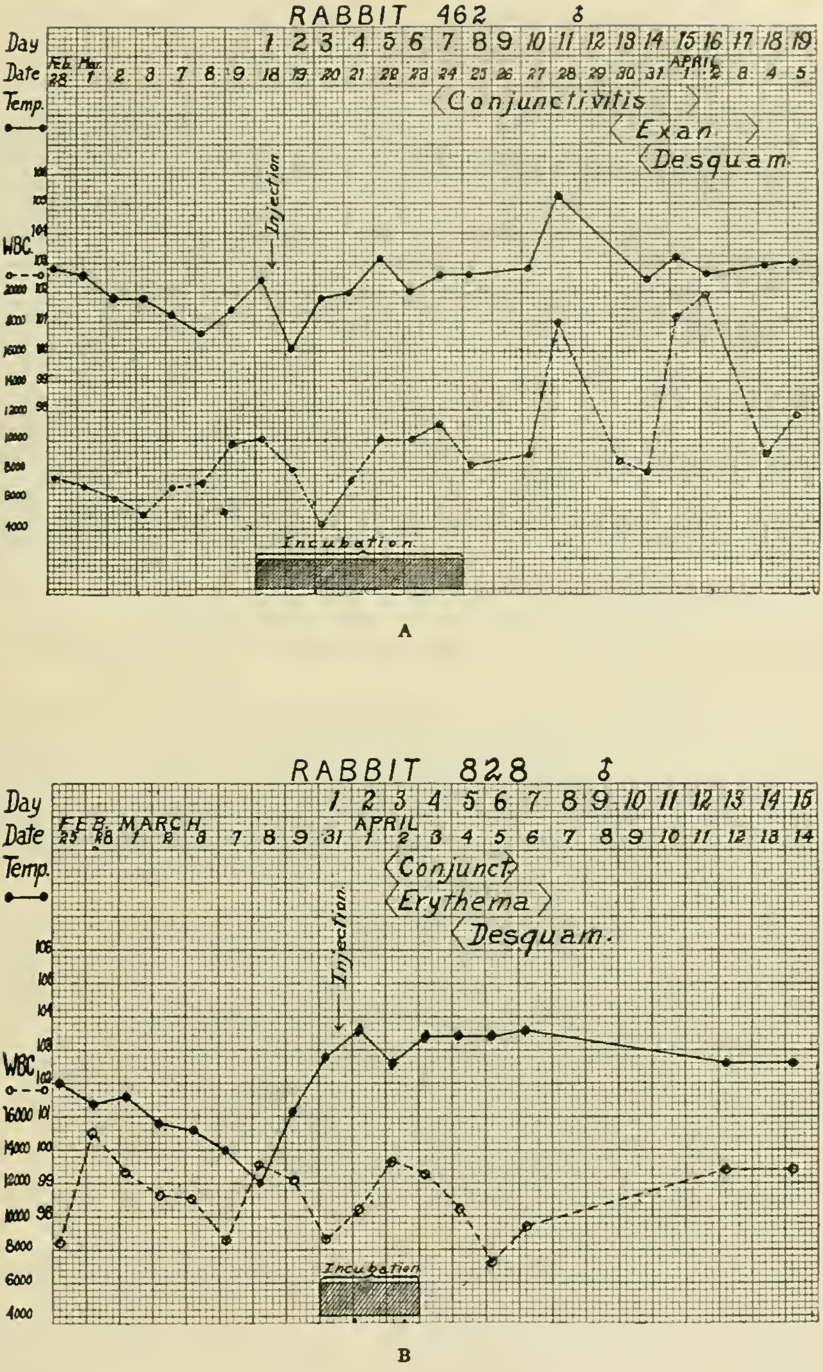


Chart 2.—Transmission of measles virus, strain from case "S," by means of intravenous inoculation of whole citrated blood; A, rabbit 462 from case "S"; B, rabbit 828 from rabbit 462.

(Reprinted by courtesy of The Journal of Infectious Diseases.)

In order to test the receptivity of the monkey to the infection of measles with blood from a human being with measles the following experiment was made:

Two patients with measles, "Y" and "O," were bled on the third day after the onset of the disease. In both cases Koplik spots were present. Conjunctivitis and coryza were present in one case only. Both cases showed a maculopapular rash. The citrated blood of these two patients was pooled and 10 cc. was inoculated intravenously within an hour into monkey 26, M. rhesus (chart 5B). On the fourth day after inoculation the animal was drowsy; loss of appetite was noted, and the diminution in the total leukocyte count was marked. The monkey developed no distinct symptoms until the sixth day when a few bright red spots, slightly elevated, were noted on the labial mucosa. On the seventh day discrete, slightly raised, hyperemic macules with bluish white centers were present on the inside of the cheeks; the enanthem on the labial mucosa was more marked but not coalescent. On the eighth day we noted a few flat reddish macules, disappearing under pressure, on the face and shoulders. Conjunctivitis appeared on the ninth day; the lids were thickened and hyperemia and lacrimation were present. The following day the exanthem had spread to the chest and abdomen, while the rash on the face of the monkey showed a yellowish brown pigmentation. During the next 4 days the exanthem gradually faded, the spots on the chest and abdomen becoming a yellowish brown also. Desquamation started on the eighth day, but was never marked. By the fourteenth day the animal appeared well; the exanthem had entirely faded and only a slight pigmentation and desquamation were noted.

Controls.—Eleven rabbits were shaved over the neck, chest, axillae, abdomen and groin. Six rabbits were inoculated intravenously each with 5 cc. of citrated blood as follows: two with blood from two cases of diphtheria, one from a case of lobar pneumonia, one from a case of lethargic encephalitis, one with normal human blood and one with normal rabbit blood. The total leukocyte count of all these rabbits showed considerable variation from day to day with the exception of the rabbit inoculated with normal rabbit blood. This count remained fairly constant.

The 5 uninoculated rabbits were kept under observation for the appearance of an erythema and desquamation. All of the 11 control animals showed a redness of the skin incident to shaving. This flush disappeared in from 48 to 72 hours whereas, in the animals showing evidence of a reaction to the inoculation of blood from patients with measles, the redness became more marked from the third to the eighth day following the inoculation and persisted for from 3 to 5 days and then gradually faded. In no instance was desquamation noted in the 11 control rabbits.

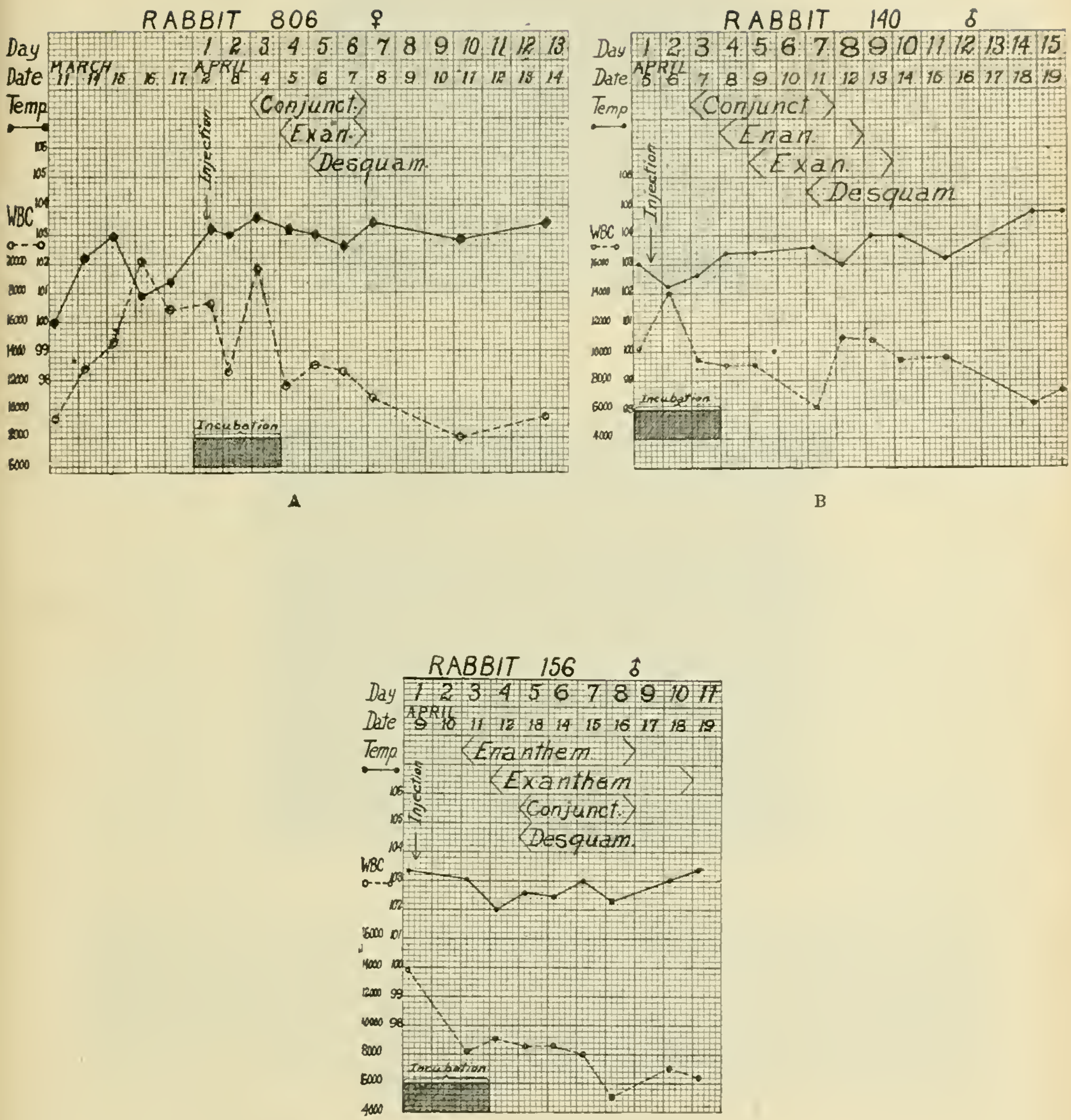
Microscopic examination of scrapings made from the spots on the labial mucosa showed no parasitic invasion.

Aerobic and anaerobic cultures made of blood prior to inoculation showed no evidence of growth.

SUMMARY

Blood from 6 patients with measles was inoculated into rabbits. The animals gave evidence of infection.

Passage from one human case of measles was carried on through 5 rabbits, and a monkey inoculated with the blood of the fifth rabbit gave typical symptoms of measles.



A

B

C

Chart 3.—Transmission of measles virus strain from case "S" by means of intravenous inoculation of whole citrated blood; A, rabbit 806 from rabbit 828; B, rabbit 140 from rabbit 806; C, rabbit 156 from rabbit 140.

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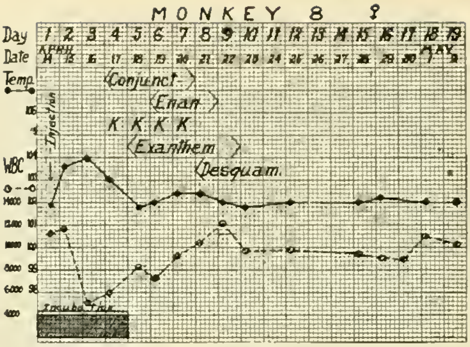
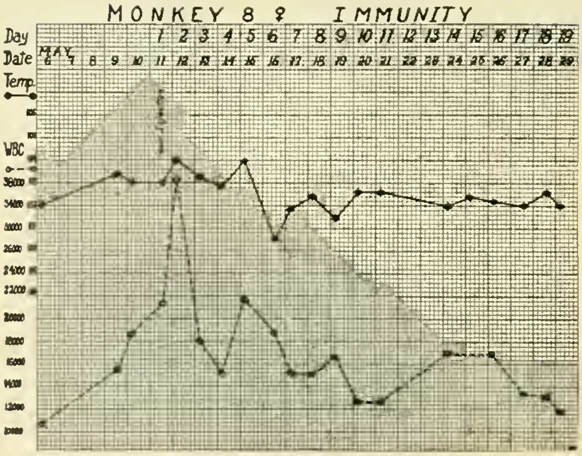


A



B

Chart 4.—Observations on monkey 8; A, transmission of measles virus strain from case "S" by means of intravenous inoculation of whole citrated blood from rabbit 156 (chart 3C); B, attempted reinfection by means of intratracheal inoculation with 10 cc. of nasopharyngeal washings containing another strain of measles virus (case "O"). The rise in the total leukocyte count prior to inoculation was due, in all probability, to a slight diarrhea. This condition rapidly ceased on change of food. As Blake and Trask have noted, the sharp rise following the injection of nasopharyngeal washings is probably due to the effect of other organisms. This animal showed no reaction to the virus and was discharged on the nineteenth day. Control monkey 24 (chart 5A), inoculated with 10 cc. of the same washings developed the characteristic symptoms of measles in 6 days.

(Reprinted by courtesy of The Journal of Infectious Diseases.)

A monkey inoculated with pooled blood from two human cases of measles taken on the third day after the onset of the disease gave the characteristic symptoms of measles.

Blood from cases other than measles, when inoculated into rabbits, failed to give evidence of infection.

For bibliography see the original article.

FURTHER NOTES ON EXPERIMENTAL MEASLES IN RABBITS AND MONKEYS

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Mary Nevin and Florence R. Bittman

During the past winter confirmation of our work on experimental measles in rabbits and monkeys[1] was undertaken. Blood from 3 early cases of measles was used for the inoculation of 2 series of animals. The blood was drawn on the second day of the disease from patients with measles in the Willard Parker Hospital for Contagious Diseases and within an hour was injected into the animals.

Case Histories

Patient Z: Ill 2 days, coryza, purulent conjunctivitis, Koplick spots, general maculopapular rash.

Patient Q: Same symptoms as patient K.

Patient Z: Ill 2 days, coryza, purulent conjunctivitis, Koplick spots, general maculopapular rash.

Series 1.—The bloods of patients K and Q were drawn on the same day, pooled and inoculated into 1 monkey and 3 rabbits (chart 1). The inoculations were intravenous, and citrated blood was used.

Monkey 31 (M. rheus) received 12 cc. of blood and developed a leukopenia on the fourth day; this was followed on the next day by an enanthem. Then Koplik spots developed and on the sixth day a general maculopapular rash was noted. A fine branny desquamation began on the ninth day.

Three rabbits (1422, 1423, 1425) inoculated with 3.5, 4 and 5 cc. respectively, developed reactions on the fourth and fifth days. The reaction consisted of lacrimation, erythema and exanthem followed by desquamation. One of these rabbits (1422) was bled on the eighth day after inoculation and 3 rabbits (1432, 1433, 1438) were inoculated with 4, 6 and 7 cc. respectively, of the citrated blood. On the second day rabbit 1438 developed an enanthem which lasted for 4 days. The entire labial mucosa was hyperemic, the lips thickened and swollen and the lower labial mucosa intensely injected. No Koplik spots were noted. The exanthem appeared on the eighth day and was followed by a flaky desquamation. No enanthem was noted in the other 2 rabbits, but the exanthem which appeared on the fourth and seventh days was marked. A general maculopapular rash was present on the chest, axillae, and groin; brownish pigmentation and large flaky desquamation followed on the eleventh day.

Two rabbits (1444, 1448) were inoculated with 6 and 7 cc., respectively, of citrated blood drawn from rabbit 1438 on the eighth day. Both of these animals developed reactions, and rabbit 1444 was bled on the fifth day.

Rabbit 1454 inoculated in turn with 10 cc. of this blood showed a marked erythematous rash on the third day. From this animal, 10 cc. of blood was drawn on the fourth day and inoculated into monkey 86 (M. rhesus). A leukopenia occurred on the fourth day. On the following day an enanthem was noted on the upper and lower labial mucosa. No other symptoms were noted until the eighth day when a marked exanthem appeared on the face, neck and shoulders; the rash spread to the abdomen and groin, and gradually faded; desquamation commenced on the tenth day.

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[1] Jour. Infect. Dis., 1921, 29, 4, p. 429.

As seen in chart 1, these typical symptoms in the monkey developed after four rabbit passages.

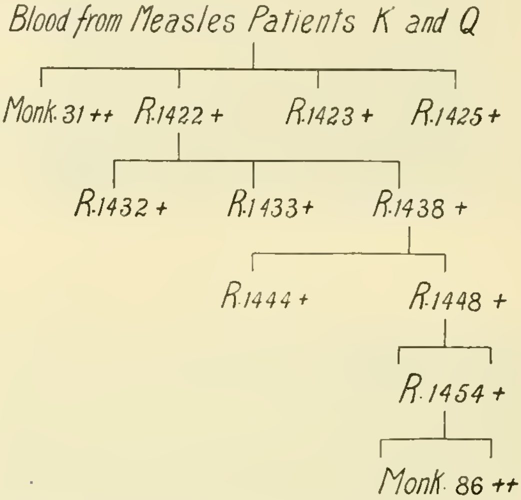

Chart 1.—Transmission of measles virus from measles patients K and Q through four rabbits to a monkey. Suggestive reaction +; typical reaction ++.

(Reprinted by courtesy of The Journal of Infectious Diseases.)

Series 2.—Measles virus from the blood of patient Z (chart 2) was successfully carried through 3 rabbits and 3 monkeys (M. rhesus) without any apparent diminution in its ability to produce typical measles symptoms in the monkey. Our idea in passing the virus from monkey to monkey at the end of the experiment was to eliminate as far as possible all question of a serum rash caused by the injection of blood from a different species of animal.

Citrated blood in amounts varying from 2.5 to 10 cc. was used throughout, with the exception of the last passage to monkey 3 when defibrinated blood was used. All inoculations were intravenous. The symptoms are recorded in the chart.

In order to note the appearance of the rash, all of the rabbits in both series were shaved the day before injection. Our previous experience had shown that careful shaving of rabbits produces no lasting irritation which would be confused with a true erythematous rash. White blood counts were made on all animals. In the case of the rabbits, the results confirmed our previous findings that the white blood count of these animals is inconstant and cannot be relied on as a diagnostic point in rabbits.

Controls.—In our previous work we had used 11 rabbits as controls, giving them intravenous injections of blood from diseases other than measles (diphtheria, lobar pneumonia, lethargic encephalitis), also normal human blood and normal rabbit blood. As none of these rabbits showed any reaction to these injections, we did not deem it necessary to repeat this part of the work.

A normal monkey was, however, injected intravenously with 5 cc. of normal defibrinated human blood and gave no reactions to the injection. Daily blood counts on this animal showed no leukopenia nor did an enanthem or exanthem develop.

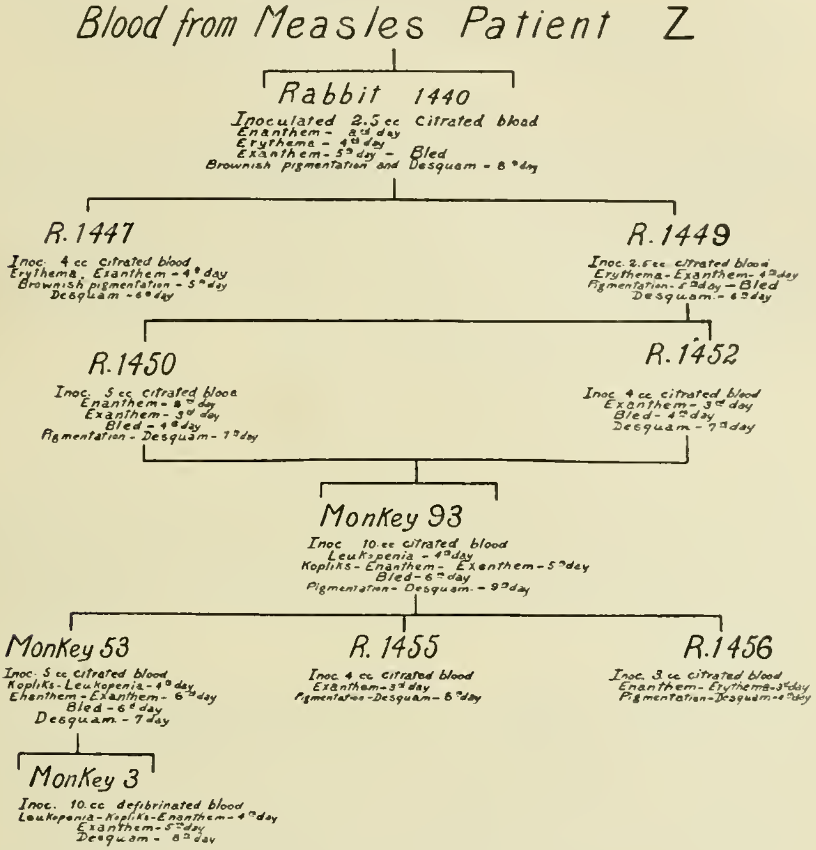
Blood from Measles Patient Z
Rabbit 1440
Inoculated 2.5 cc citrated blood
Enanthem - 3rd day
Erythema - 4th day
Exanthem - 5th day - Bled
Brownish pigmentation and Desquam - 8th day
R. 1447
Inoc. 4 cc citrated blood
Erythema, Exanthem - 4th day
Brownish pigmentation - 5th day
Desquam - 6th day
R. 1449
Inoc. 2.5 cc citrated blood
Erythema - Exanthem - 4th day
Pigmentation - 5th day - Bled
Desquam - 6th day
R. 1450
Inoc. 5 cc citrated blood
Enanthem - 2nd day
Exanthem - 3rd day
Bled - 4th day
Pigmentation - Desquam - 7th day
R. 1452
Inoc. 4 cc citrated blood
Exanthem - 3rd day
Bled - 4th day
Desquam - 7th day
Monkey 93
Inoc. 10 cc citrated blood
Leukopenia - 4th day
Kopliks - Enanthem - Exenthem - 5th dey
Bled - 6th day
Pigmentation - Desquam. - 9th day
Monkey 53
Inoc. 5 cc citrated blood
Kopliks - Leukopenia - 4th day
Enanthem - Exanthem - 6th day
Bled - 6th day
Desquam. - 7 day
R. 1455
Inoc. 4 cc citrated blood
Exanthem - 3rd day
Pigmentation - Desquam - 6th day
R. 1456
Inoc. 3 cc citrated blood
Enanthem - Erythema - 3rd day
Pigmentation - Desquam - 4th day
Monkey 3
Inoc. 10 cc defibrinated blood
Leukopenia - Kopliks - Enanthem - 4th day
Exanthem - 5th day
Desquam - 8th day

Chart 2.—Transmission of measles virus from measles patient Z through three rabbits and three monkeys. (Reprinted by courtesy of The Journal of Infectious Diseases.)

SUMMARY

In the first series of animals the virus of measles obtained from patients with measles on the second day of the disease was passed through 4 rabbits and then produced symptoms typical of measles in a monkey.

In the second series of animals the virus obtained as stated was passed through 3 rabbits and then through 3 monkeys in which symptoms typical of measles were produced. The monkey to monkey passage eliminated any question of rash due to foreign protein.

A control monkey injected with normal human blood showed no reaction.

CONCLUSION

The results of these two series of passages confirm our previous findings that the virus of measles survives rabbit passage and produces symptoms typical of measles in the monkey (M. rhesus).

GROWTH OF B. INFLUENZAE WITHOUT THE PRESENCE OF HEMOGLOBIN

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Anna W. Williams and Olga R. Povitzky

An observation made years ago by Cantani and corroborated by Neisser and others, including ourselves, has recently been denied by Putnam and Gay. The observation is that the influenza bacillus will grow in mixed cultures with certain bacteria without the aid of hemoglobin. Notwithstanding a number of earlier negative reports, positive results had been reported by so many workers and had been obtained by ourselves after so many repeated controlled experiments that we considered that this was one of those questions that might be accepted as settled. Putnam and Gay, however, do not agree with us and neither apparently does Rivers.

The first named authors, Putnam and Gay, do the very thing they criticize in others: namely, they give too few details for us to be sure of the reason why they obtained their negative results.

In the first place, they do not state the age of their cultures at the time of inoculation, neither of the influenza bacillus (which is of special importance), nor of the organisms used for stimulation. Presumably, the age was forty-eight hours, as that is supposed to be the period of maximum growth of most bacteria at 37°C. Anything over that age, according to our experience, is likely to give irregular results with influenza bacilli. We might infer, however, considering their negative results, that these workers used seventy-two hour cultures.

In the second place, they do not state clearly enough the amount of culture they inoculated originally to produce their mixtures. They say that they inoculated rather lightly with the second organism used. We have found in growing certain organisms in mixtures, that unless we inoculate *very* lightly with the stimulating organism in the first culture generation, the influenza bacillus is likely to be overgrown by the accompanying organism.

Another reason for the negative results of Putnam and Gay may be that their culture medium was too alkaline. We have found a reaction of Ph. 7.2—7.4 best for the growth of the influenza bacillus. These authors say that they used a medium varying in reaction from 0.2 to 1.2 acid to phenolphthalein. Just what their end reaction was in each lot of medium we do not know. However, we do know that the reaction of medium is a very important point in relation to the growth of the influenza bacillus. While these bacilli may grow well on certain favorable media at the extremes of reaction, under unfavorable conditions there may be no growth at all at these extremes. We consider 0.2 per cent. acid to phenolphthalein as approaching the extreme towards alkalinity in unfavorable media.

In the fourth place, the medium used may have been so favorable for the stimulating organism that it readily overgrew the influenza bacillus or it may have produced substances deleterious to the influeza bacillus. For example, our cultures mixed with diphtheria bacilli on glucose agar were quite unsatisfactory, while on plain veal agar and on wheat agar the influenza bacilli grew very well in the mixture. Putnam and Gay used glucose agar for their diphtheria and xerosis mixtures.

The authors state that, theoretically, minute traces of blood sufficient for the growth of the influenza bacillus may gain access to the medium through the meat infusion ordinarily employed but if the negative results reported can be relied upon, this can occur seldom, if ever. Putnam and Gay call attention to the fact that Ghon and von Preyss suggested this possibility in 1902. Their idea seems to be that though there would not be enough hemoglobin to allow the growth of the influenza bacillus in pure cultures, a stimulating organism plus the minute trace of hemoglobin would allow growth. Rivers seems to agree with this theory. The interesting work of Thjötta and Avery, elaborating in part the work of Davis, seems to come quite near the solution of the problem of the relationship of hemoglobin to the influenza bacillus. They state that pure crystalline hemoglobin does not by itself support growth but contains only one of the two essential factors for the growth of the influenza bacillus. One factor, "V" is destroyed by autoclaving, the other factor, "X" is not. Avery has other data* which shows that these two factors are found in substances other than a solution of non-autoclaved hemoglobin. His data give evidence also of the nature of these two substances. Whether the bacteria which allow growth contain substances identical with "X" and "V" has not yet been shown.

Experimental Work

Having on hand a number of strains of *B. influenzae* some of which we had isolated during the great pandemic, we thought it might be interesting to repeat our work on mixed cultures of B. influenzae by growing some of these strains with certain organisms on a medium in which no trace of hemoglobin could be present. In order to meet this condition we employed a medium which was made from wheat and contained no meat extract, of any kind. The formula and directions for making are given in the original paper.

This wheat medium was recommended for general laboratory purposes by S. Otabe and was prepared by Dr. Jane Berry of our laboratory for our work. She had made extensive tests of its ability to grow the gonococcus and the other strains of "stimulating" organisms we were using and had pronounced the results ve y good.

In the first series of experiments, three other kinds of mediums were used besides this wheat medium (see table in original article)—nutrient veal agar made according to the formula given in Park and Williams, the same agar plus 1 per cent. glucose sterilized in Arnold, and "chocolate agar" as one control. The final reaction of all these media was not beyond the limits of Ph. 7.4—7.2. The regular transplants were made every forty-eight hours on about 6 cc. of slanted medium in test tubes, 15×3 cm. At the time of each of these transplants additional transplants were also made on Avery's oleate blood agar plates and on veal blood drop plates in order to get surer evidence than could be obtained from stained spreads as to the presence of living influenza bacilli. Avery's medium, as usual, did good service in demonstrating influenza bacilli in mixed cultures with Gram positive bacteria. We had, however, to make the proper dilutions with the cultures containing the yeast-like organisms which we used, because it would readily overgrow the influenza bacilli, making their demonstration difficult if not impossible. The differentiation between the colonies of B. diphtheriae and B. influenzae was best made on the veal-agar-blood-drop plates.

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* Personal communication.

History of strains chosen for this study.—Six strains of B. influenzae were used and five strains of "stimulating" organisms.

Strains of influenza baccilli:

1. "Pembroke,"—from an autopsy case isolated in 1918, as a pure culture from the lungs. It is an individual type by agglutination.
2. "Trahan," another autopsy strain isolated in 1918 and agglutinated individually.
3. 6747,—from a spinal fluid in case of influenzal meningitis in autumn of 1918, agglutinating individually.
4. "Z,"—from another meningitis spinal fluid isolated in 1912. Serologically it belongs to same group as the following two.
5. 747,—from spinal fluid of another meningitis case, isolated in 1912. Serologically it belongs to same group as Z.
6. "B. I. W."—from spinal fluid of case of meningitis isolated a number of years ago by Dr. Wollstein.

Strains used as stimulating organisms:

1. B. diphtheriae,—our No. 8 which we use in making our diphtheria antitoxin, isolated in 1895.
2. "Streptococcus hemolyticus," No. 270, isolated by Valentine in 1917 from a case of mastoiditis.
3. "Gonococcus,"—from one of Torry's strains, "L," used by us in our diagnostic work.
4. "Monilia" (species undetermined),—isolated by one of us in 1920 from the tongue in a case diagnosed as sprue.
5. "Staphylococcus aureus,"—isolated by Valentine from a boil several years ago and used as one of our routine cultures.

We chose the autopsy and meningitis strains of B. influenzae because we had studied them more closely than many of the others. They had all been classed by us as typical influenza bacilli, and they have the following characteristics: All appear to be strict hemoglobinophiles in pure culture; they grow in characteristic colonies on blood drop veal agar plates, and on Avery's oleate agar plates; they have a characteristic morphology in young cultures, namely, they are minute Gram negative bacilli with a tendency to form threads, especially on certain culture media; no one of them is hemolytic; Povitzky found that two of the meningitis strains ("Z" and "747") produce indol; the strains from meningitis sources produce a slight amount of acid in glucose media in forty-eight hours; none produces gas; Strain Z is more virulent for animals than any other strain tested.

For one of the stimulating strains we used B. diphtheriae rather than B. xerosis because in our earlier work B. influenzae in mixtures with this organism had not grown quite so abundantly as with the xerosis bacillus and we wished to make the test as rigorous as possible. For the same reason, we chose a hemolytic streptococcus rather than a pneumococcus or other green-producing streptococcus as in the many times we had made this test with the group of Gram positive streptococci, we had found that all of the stains of influenza bacilli tried, grew better in mass cultures with the green producing streptococci than they did with the hemolytic streptococci which we used. So we again chose the species which had given us the least abundant growths of the influenza bacillus in these earlier experiments. However, we chose the gonococcus as a contrast to the other two because with it we had found that the influenza bacillus grew most abundantly. Cantani was the first to use gonococcus in mixed cultures with influenza bacilli. He discovered accidentally that the gonococcus was a good sensitizing organism and he made some interesting experiments at that time.

We used the monilia because previously we had obtained such good results with yeast, and this monilia was the only yeast-like organism we had growing at this time. (Povitzky succeeded earlier in growing B. influenza with Baker's yeast for many generations.) We chose Staphylococcus aureus as another contrast because we had never obtained growths of the influenza bacillus when put in mixed culture with it, beyond the first few culture generations.

Factors to be considered in carrying out this work successfully: 1. A medium should be used that is not too favorable for the growth of the stimulating organism and that does not produce substances unfavorable to the influenza bacillus. Thus, in our first series of transplants (see table in the original paper) of the mixture of influenza with B. diphtheriae on glucose veal agar, four of the six influenza strains dropped out on the second culture generation and the other two on the seventh and ninth generation respectively, while on plain veal agar all six strains went on growing vigorously through twenty-two culture generations (no more generations tried).

Whether the dropping out of the influenza bacillus from the diphtheria mixtures grown on glucose veal agar, was due to the production of too much acid rather than to a too vigorous growth of the diphtheria bacillus, or to another cause or to a mixture of causes, we do not know. The production of acid is possibly the largest factor. Whatever the cause may be it was probably the same as that giving Putnam and Gay's negative results with their diphtheria and xerosis mixtures.

2. The second factor to be considered in making these transplants is the converse of the above, that is, the medium must be favorable enough for the stimulating organism. For example, when this first series was started the wheat medium used grew the gonococcus quite well through the fourth culture generation. At this time that particular lot of wheat medium was nearly used up and another was employed which gave such a scanty growth that none of the mixtures grew on it after the second or third culture generation. This lot was made during Dr. Berry's absence and the directions could not have been followed absolutely. When Dr. Berry returned, she made up fresh medium which gave most successful growths. With this we started another series of mixtures the results of which are given in the parentheses in the table. We had saved enough of the original medium to carry the mixture of B. diphtheriae, and B. influenzae (strain "Trahan") through twenty-two culture generations or up to the time this series was stopped.

3. The third factor to be considered is the amount of each culture transferred at the first transplant when the mixtures are made. After the first transplant it does not seem to make any difference, within the limits tried, how much or how little of the mixtures is transferred, but when the two cultures are first put together if more than an extremely small amount of certain of the stimulating organisms is used they are likely to overgrow the influenza bacillus. For this reason, we inoculated the gonococcus and the hemolytic streptococcus with a small platinum loop (2 mm.), whereas we transferred the diphtheria bacillus, the monilia and the staphylococcus by touching only the tip of a fine platinum needle to the culture to be transferred. Even with this small amount, the staphylococcus seemed to overgrow the influenza bacillus quickly.

4. The fourth factor is the age of the cultures when transferred. As a rule, all of our cultures were forty-eight hours old when transplanted. Younger cultures we did not try. If the influenza cultures were over forty-eight hours old when transplanted for the mixtures, they were more easily overgrown by the stimulating organism and they seemed unable to recover

their original vigor. For example, on repeating successive transplants on plain veal agar of a mixture of B. influenzae (using three strains respectively) with B. diphtheriae, the strain B. influenzae (Trahan) which had grown the best in the other series, grew much less vigorously than in the previous mixtures. This strain when transplanted for this second series was seventy-two hours old, whereas the companion cultures were forty-eight hours old just as in the first series.

The following observations may be made on this work:

1. Certain strains of B. influenzae grew well on nutrient veal agar in mixed cultures with each of the stimulating organisms tried except the staphylococcus. All grew well with the diphtheria bacillus and the gonococcus. All but one grew well with the monilia. Three only grew well with the streptococcus. These influenza strains did not grow in pure cultures when planted on this same veal agar neither did they grow on this agar when extracts of the gonococcus were added to it (see below), so presumably this medium had none of the "X" factor of Avery and Thjötta.

2. On glucose veal agar, contrary to the above, B. influenzae continued to grow through the entire series only in combination with the gonococcus. With the monilia, the succession continued nearly to the end but with all of the other stimulating organisms the influenza strains dropped out with varying quickness as indicated in the table in the original article. We have mentioned the possible causes of this lack of continued growth.

3. On wheat agar, as we have already said, when the medium was prepared correctly, the influenza bacilli grew abundantly with both diphtheria bacilli and gonococci. Presumably they would have done the same with the streptococcus and monilia; but, judging from the way they acted on other media, no continued growth with staphylococci could be expected.

4. On chocolate agar (which was considered one of the controls) it is interesting to note that while the influenza bacilli seemed completely to overgrow the gonococcus and tended to overgrow the diphtheria bacillus, they were more or less quickly overgrown by the streptococcus and the staphylococcus. In our previous work we had obtained the same results with the pneumococcus as with the gonococcus, that is, the pneumococcus disappeared from the mixture after a certain number of culture generations. Under all the conditions so far tried, the influenza bacilli have disappeared from the staphylococcus mixtures very early. That living staphylococci may contain the factors necessary for the growth of the influenza bacillus is possible, and more experiments may show that the lack of growth is due to an inhibiting factor rather than to an absence of the required factors.

5. Not only do we get varying results on the different mediums with the different stimulating organisms but on the same mediums with the same stimulating organism we get somewhat varying results with the different strains of influenza bacilli. Thus, in the first series of transplants Trahan grew more abundantly on all the mediums used than did any other strain, while "Z" was the poorest grower and the other two strains showed variations between these extremes. These variations were shown chiefly in the mixtures with streptococcus and to a less extent with the monilia.

6. The agglutinating properties of the strains of B. influenzae remained unchanged after their successive transplants in mixed cultures.

Attempts to grow B. influenzae with killed cultures of the stimulating organisms or with their extracts.—Cantani and Luerssen claim to have grown B. influenzae on blood-free mediums with killed cultures of bacteria. Neither of them mentions the number of culture generations through which the growth was carried on. Neisser, on the contrary, though he reported the successful growing of B. influenzae with living cultures of B. xerosis on blood-free agar through twenty culture generations, said he could not corroborate Cantani's work with killed cultures. He assumed, therefore, that a special fermentative assimilation of the foodstuffs by the living organism during its growth is essential for the stimulation of the growth of B. influenzae on blood-free mediums. He gave the name "Amme" (wet nurse) to the stimulating organism.

If neither of the two factors said by Thjötta and Avery to be necessary for the growth of the influenza bacillus is contained in the blood-free nutrient media used by us and if dead bacteria or their extracts contain only one of these factors, as Thjötta and Avery state, then no growth could be expected on a medium made up of a blood free medium and dead bacteria or their extracts.

First experiment.—Monilia and gonococci were killed at different temperatures. The first emulsions of killed organisms tried were prepared as follows:

> Forty-eight hour cultures of gonococci or of monilia grown on wheat agar were washed off with salt solution (0.85 per cent.) and the emulsion was heated at 60° C. for one, two, or three hours. The emulsion was then incorporated with the melted wheat agar in the tubes, cooled down to 60° C. Some tubes were poured into plates, others were slanted.

These tubes and plates were then inoculated with B. influenzae.

Extracts of gonococci or of monilia were added to half the tubes and plates on the surface together with the culture of B. influenzae. Proper controls of tubes and plates were made with or without the corresponding extracts to test the sterility of the latter.

The extracts were prepared as follows:

> A forty-eight hour growth of gonococcus or of yeast on blood free medium was washed off with distilled water and shaken in an electric shaker for four to five hours. Half of this emulsion was placed in the ice box and half in the incubator at 37° C. over night. The next morning the emulsions were filtered through a Berkefeld filter and tested for sterility.

As a result of these efforts, growth of B. influenzae was obtained only in those instances when the controls proved that the stimulating organism had not all been killed.

In the second experiment the emulsion was prepared as follows:

> Forty-eight hour cultures of monilia or of gonococci on wheat agar medium were autoclaved. Some of the tubes were then slanted again and others were poured into plates. The tubes and plates were then inoculated on the surface with B. influenzae with or without the corresponding extracts.

No growth was obtained on any surface.

In the third experiment tried the procedure was the same as in the second one except that the cultures were arnolded instead of being autoclaved.

The results were negative.

The fourth experiment was varied as follows:

> A forty-eight hour growth of gonococcus or monilia on wheat agar slants in potato tubes was heated in a water bath at 60° C. for one hour, then inoculated with B. influenzae with or without the corresponding extracts.

The immediate results were not certain. The influenza bacillus was too much masked by the original growth on the surface. The transfers were carried on for three culture generations. At the end of these transplantings Avery's plates were negative for B. influenzae.

In order to ascertain whether or not a ferment or other substance capable of causing growth was secreted in the medium during the growth of the stimulating organism the fifth experiment was made as follows:

Forty-eight hour growths of gonococcus or monilia on wheat agar were removed by washing off with salt solution (0.85 per cent.) and the same tubes were inoculated with B. influenzae, with or without the corresponding extract.

The results were negative.

These negative results with dead cultures and their extracts are corroborative of all our earlier results in our efforts to grow influenza bacilli with dead cultures of micro-organisms. They show that at least one of the substances necessary for the growth of the influenza bacilli had been made inactive by our method of killing the stimulating organism.

Along with these attempts to grow the influenza bacillus on blood free medium with dead bacteria or their extracts we fully corroborated the work of Thjötta and Avery in regard to the growth of these organisms with pure hemoglobin or with autoclaved blood, in fluid mediums. That is, pure hemoglobin in any nutrient medium tried does not support the growth of the influenza bacillus. Neither does autoclaved blood alone support the growth in such medium. But when extracts of certain micro-organisms are added to the medium containing either of these two substances growth of the influenza bacillus is supported indefinitely on successive transplants.

Whether the substances necessary for the growth of the influenza are found elsewhere than in blood and in certain living micro-organisms is a question which has already been solved by Avery (personal communication) and may be more fully worked out in the near future, since a number of investigators are at work on the subject.

Conclusions

Influenza bacilli may continue to grow in mixed mass cultures with certain micro-organisms on the surface of certain media containing no hemoglobin, through an indefinite number of successive culture generations.

They will not grow on such media with cultures of the same stimulating organisms killed by the method we employed, or with the extracts of these organisms.

For bibliography see the original article.

Annotation: We fully corroborate the work of Avery; namely, of growing B. influenzae in broth containing small pieces of sterile fresh potato. This growth was continued for 12 to 15 generations and then discontinued. The same results were obtained with beets and potato arnolded for 10 minutes. These same vegetables failed to support growth when heated 15 to 20 minutes. Our results in growing B. influenzae with autoclaved potato to which an extract of yeast was added were, on the other hand, variable.

IMPROVED METHODS FOR THE ISOLATION AND LATER CULTIVATION OF B. PERTUSSIS

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The need for more strains of B. pertussis for inquiry as to the presence of types and the possibility of making a more effective vaccine was the starting point of this study. It was difficult to obtain material in the beginning because there were not many cases of whooping cough in New York City during the winter of 1921-1922. Those reported were usually too advanced for the isolation of the organism. We were, therefore, on the lookout for secondary cases in the families in which pertussis had been reported, but in most instances we were able to obtain sporadic cases only.

Bordet and Gengou emphasize the fact, corroborated since by many, that it is a compartively easy task to isolate B. pertussis in the early stages of the disease. In the first ten days under favorable conditions, an almost pure culture of B. pertussis is frequently obtainable.

In general, the favorable factors include: (1) a sputum which has been obtained at the end of the characteristic spasmodic cough; (2) the washing of the sputum a few times in salt solution, after which a suspension for plating is made of any characteristic balls present; (3) freshly prepared plates of Bordet-Gengou medium made with defibrinated or citrated blood in the proportion of 1:3. The blood must be added at a temperature of from 45 to 50 C. and well mixed with the agar so that the plates will appear smooth, uniform and of a proper red color.

It is hard to get sputum from young children unless it is expelled in a fit of spasmodic coughing, or is removed from the mouth or throat with a spatula by depressing the tongue before the child has had a chance to swallow after the coughing spell.

The medium in use in this laboratory for isolation of B. pertussis is that of Bordet-Gengou modified slightly in a few points: thus 2% instead of 1% glycerol is added; horse defibrinated or citrated blood instead of human or rabbit blood is used. The original formula of Bordet-Gengou does not give the reaction of the medium. Looking over the literature on the isolation of B. pertussis, we find that Arnheim is the only one who mentions the reaction which he says is feebly acid, but he does not say which indicator he used to ascertain this point. The unadjusted Bordet-Gengou medium is feebly acid to litmus, but when tested as to the H-ion concentration, it is found to be Pн 5.8 to 6.1, that is, rather acid. In our laboratory the medium was originally adjusted by adding 8 cc. of normal sodium hydroxid to each liter; this made the reaction about neutral to phenolphthalein before sterilization and supposedly slightly acid after sterilization. Williams, Wilson and Mishulow adjusted their Bordet-Gengou medium to this reaction for the isolation of B. pertussis in 1912 (personal communication). Recent tests of such a medium have shown that it may vary in reaction from Pн 7 to 7.8; usually it is about 7.4 to 7.6. In the beginning of the present work, we used a Bordet-Gengou medium adjusted to 7.2 to 7.4.

TECHNIC

From 6 to 8 plates of Bordet-Gengou medium and 3 "chocolate" plates made with Avery's sodium oleate agar were used for each case. The latter were useful for the purpose of determining the percentage of B. influenzae present. The Bordet-Gengou plates were incubated for from 48 to 72 hours and occasionally for 4 days. The colonies picked for fishing into Bordet-Gengou tubes were the minute whitish colonies, preferably those which showed a lightened area of the surrounding medium. In 48 hours, smears were made from all pertussis-like cultures (usually a whitish nonspreading growth), stained by gram and examined under the microscope. If the smears showed small ovoid, uniform gram-negative rods, the culture was transplanted to both chocolate and plain agar. If no growth occurred on either of these agars and if at the same time a good growth occurred on the Bordet-Gengou medium, the growths were considered culturally B. pertussis. They were then turned over to Miss Mishulow for further identification and typing with known pertussis serums.

In most cases from which B. pertussis was isolated the culture was obtained pure at the first attempt, but occasionally it was necessary to replate it a second or third time.

For these cases Bordet-Gengou medium (reaction PH 7.2 to 7.4) was used exclusively. In this series we succeeded in obtaining 8 isolations of B. pertussis. The period of the disease in which the successful isolations occurred varied from the first to the third week of the whoop: in the first week, 3 isolations of 7 cases; in the second, 4 of 14 cases; in the third, 1 of 7 cases.

The cases were usually in the postcatarrhal stage. The statements of the patient's family were not always reliable, for the parents were not clear as to the distinction between the beginning of the cough and the beginning of the whoop.

This series comprised uncomplicated whooping cough only. Cases complicated with pneumonia, bronchopneumonia and exanthems are not included. Neither are any suspicious cases in which the patient developed a cough after a course of prophylatic treatment with pertussis vaccines. These children did not prove to have pertussis eventually. In neither of these groups was B. pertussis isolated.

FIRST SERIES; 35 CASES (TABLE 1)

TABLE 1

Isolation of B. Pertussis on Bordet-Gengou Medium, Reaction Ph 7.2-7.4

First Series of Thirty-Five Cases

Number of Cases	Week of Whoop	Number of Isolations
7	1	3
14	2	4
7	3	1
7	4	0

The percentage of successful results obtained with our first series, namely, 8 isolations from 35 cases (over 22.8%) correspond more or less with the results of most other workers, with a few exceptions (see table 2). In contrast to the majority of workers, Menschikoff reported almost 100% of isolations up to the eighth week of the disease; Inaba from 88 to 91% Seiffert, early cases, 75%; Chievitz and Meyer, from 53 to 41%. The first two workers do not state whether they verified their cultures by serologic tests for complete identification.

TABLE 2

Tabulation of Isolations of B. Pertussis Reported by Various Authors

Author and Year	Number of Whooping-Cough Cases	Stage	Isolations	
			Number	Percentage
Arnheim, 1908	20	Not mentioned	6	30
Fraenkel, 1908	38	Not mentioned	8	21
Seiffert, 1909	16	Early	12	75
Wollstein, 1909	20	From catarrhal to seventh week	5	25
Williams, 1912	187	From first to fourth week	27	14½
Poleff, 1913	14	Not mentioned	3	14⅓
Odaira, 1911	42	Not mentioned	5	12
Inaba, 1912	78	Early	68	88
Menschikoff, 1909	94	From beginning till eighth week	93	Nearly 100
Klimenko, 1908	76	....	5	6⅓
Chievitz and Meyer, 1916 (1st series)	126	From catarrhal to fifth and sixth weeks	67	53
Chievitz and Meyer, 1916 (2nd series)	165		69	41.8
Shiga, 1913	Not given	Not mentioned	10	26.3
Giese, 1918	19	Not mentioned	5	

Menschikoff used Bordet-Gengou medium to which hemoglobin in the proportion of 1:3 was added. The hemoglobin was obtained by freezing and thawing the blood and keeping it at a temperature above 0 C. for 24 hours.

Inaba's medium was a plain 3% agar solution to which difibrinated goat blood was added, 1:4. He does not mention whether he used salt or water with the agar and omits reference to the reaction.

Shiga isolated 10 cultures of B. pertussis; he does not state from how many cases. His medium was a modified Bordet-Gengou, that is, 1% peptone was added and the reaction made slightly alkaline (to what?). After the blood was added, the tubes or plates were heated at 56 C. for one-half hour. This medium is so favorable for the isolation of B. influenzae that it is hard to understand how Shiga obtained pure cultures of B. pertussis. Perhaps this is the reason why this worker thinks that the two organisms are closely related and belong to one group.

Arnheim isolated 6 pertussis cultures from 20 cases and Frankel 8 from 38 cases. Frankel also reports that he isolated B. pertussis from 2 cases which were not pertussis clinically. Wollstein had 5 isolations from 20 cases. Williams, Wilson and Mishulow had 27 isolations from 187 cases (14.5%) [unpublished], Poleff 3 isolations from 14 cases, Odaira 5 isolations from 42 cases, Klimenko 5 isolations from 76 cases, and Giese 5 isolations from 19 cases.

An analysis of the 8 isolations in our first series, brings out some facts which are interesting, for they illustrate the importance of the stage of the disease in attempts to isolate the bacillus.

The first culture of B. pertussis was isolated from a child of 18 months. A sister of 5 years and a brother of 6 were reported to us as having whooping cough. These 2 children had been whooping for over 2 weeks and the results with the sputum were negative. On a visit to the home of this family, it was discovered that the little sister of 18 months had just started to whoop. There was no difficutly in isolating the organism from the sputum. Of 5 cases in a school for colored children, B. pertussis was obtained in the case of one child only—a boy of 7. On a visit to the family of one of these children, a child of 2 was found to be coughing and had just begun to whoop.

This case had not been reported to us. The sputum of this child gave us our third B. pertussis culture.

The fourth case which gave positive results was reported as suspicious whooping cough chiefly because the sister had the disease in a late stage. In the case reported, that of a girl of 10, there was a dry cough. No culture was obtainable by the droplet method recommended by Chievitz and Meyer. This method consists of making the patient cough on a Bordet-Gengou plate held a few inches from the face. In a few days the dry cough loosened up and an almost pure culture of B. pertussis was obtained from the sputum seeded on Bordet-Gengou medium.

The fifth culture was isolated from a boy of 3 years. No sputum could be obtained in the first attempt. Three days later sputum mixed with fresh vomit was brought to us; from this material we succeeded in isolating B. pertussis. A second culture taken one week later from this patient proved to be negative. The sixth and seventh cultures of B. pertussis were obtained from children in the Bellevue Clinic by the courtesy of Dr. Chaplin. One child was in the first stage of the disease, the second in the third week. The sister and brother of the first child who were in more advanced stages of the disease gave negative results. The eighth and last culture of this series was obtained from 1 of the 4 children examined in a home for children where there was an epidemic of whooping cough.

The greatest obstacle in isolation of B. pertussis was the accompanying growth of B. influenzae. The latter organism was isolated from almost every case of pertussis; it was always present in great numbers at the beginning of the second week of the whoop. The Bordet-Gengou medium with a reaction of PH 7.2 to 7.4 is favorable for the growth of B. influenzae. The colonies develop in from 18 to 24 hours and thus outgrow the B. pertussis colonies, for the latter are not apparent until the third or perhaps the fourth day of incubation. The colonies of B. influenzae are very much like those of B. pertussis, except that they do not show a light colored area in the blood plate. When, however, the colonies of B. influenzae happen to be of the hemolytic variety even this diagnostic difference disappears.

Experimental Work.—In view of the foregoing facts the chief object in the search for a more favorable medium for isolation of the Bordet-Gengou bacillus was to find a substance which would inhibit in a measure the development of the colonies of B. influenzae and at the same time be favorable for the growth of B. pertussis.

The first attempts at modification consisted in the addition of various dyes, such as gentian violet, methylene blue, methylene green, brilliant green, carmen borax and others. These were tried in different dilutions with pure cultures of B. pertussis and B. influenzae. The results were not satisfactory. The majority of these dyes acted alike on these two organisms, while a few even enhanced the growth of B. influenzae. Finally, when Grübler's acid green was tried the results were more encouraging. With a dilution of 1:20,000 of this dye, the growth of B. pertussis was much better than that of B. influenzae. The suggestion of an acid reaction in acid green prompted us to make comparative tests with the 2 organisms on a potato-glycerol-veal agar with the reaction unadjusted, and, therefore, quite acid (PH 5.8 to 6.1). We are accustomed to use this medium for the growth of B. mallei. Citrated horse blood in proportion of 1:4 was added to the medium at a temperature of 45 C., well mixed with the agar and slanted. B. pertussis and B. influenzae were inoculated in companion tubes of this medium. The tube inoculated with B. influenzae showed almost no growth after 48 hours' incubation. The growth of B. pertussis, on the other hand, was astonishing; so much so, that at the first glance, it was thought a contamination. Only a smear and a subsequent plating convinced us that we were dealing with a pure culture of B. pertussis. On this medium the Bordet-Gengou bacillus grows in a brittle pearly white mass.

The next procedure was to make comparative plate tests of B. pertussis, B. influenzae and a mixture of both in 4 mediums, 2 of these being unadjusted and therefore of an acid reaction. These mediums were: (1) Bordet-Gengou medium adjusted to Ph 7.2 to 7.4; (2) Bordet-Gengou medium unadjusted, Ph 5.8; (3) potato-glycerol-veal agar plus blood unadjusted, Ph 6. and (4) chocolate agar, Ph 7.2 to 7.4.

Uniform suspensions of the same density (rather light) were made from 48-hour cultures of B. pertussis grown on Bordet-Gengou medium and B. influenzae grown on chocolate agar; also of a mixture of both organisms. The same sized loop was used for streaking all plates. Further dilution of the suspension was made by inoculating 3 plates in succession with the same loopful.

Table 3 shows how favorably B. pertussis grows on the acid mediums as compared with B. influenzae; while on the Bordet-Gengou medium adjusted to Ph 7.2-7.4, the B. influenzae grows almost as well as B. pertussis. On chocolate agar the freshly isolated B. pertussis does not grow, while B. influenzae gives its usual luxuriant growth.

TABLE 3.

COMPARATIVE GROWTH OF B. PERTUSSIS AND B. INFLUENZAE AND A MIXTURE OF BOTH ON STRONGLY ACID AND FEEBLY ACID BORDET-GENGOU MEDIUM AND ALSO ON ACID GLYCEROL-VEAL-POTATO AGAR PLUS BLOOD

Plates			Bordet-Gengou Medium		Glycerol-Veal-Potato Agar Plus Blood Ph 6.0	Chocolate Agar Ph 7.2-7.4
			Ph 7.2-7.4	Unadjusted Ph 5.8		
B. influenzae	1		+ +	—	+	+ + +
	2		±	—	+	+ +
	3		—	—	±	+
B. pertussis	1		+ +	+ + +	+ + +	—
	2		+ +	+ + +	+ +	—
	3		±	+ + +	+	—
Mixture of B. influenzae and B. pertussis	1	B. influenzae	+	—	+	+ + +
		B. pertussis	+ + +	+ + +	+ +	—
	2	B. influenzae	+	—	±	+ + +
		B. pertussis	+ +	+ + +	+	—
	3	B. influenzae	—	—	—	+ + +
		B. pertussis	±	+ + +	±	—

In the mixture of B. influenzae and B. pertussis on the acid mediums, the preponderance of B. pertussis was made conspicuous by the areas of lighter color surrounding the colonies. There was a good growth of B. influenzae on Bordet-Gengou medium with a reaction of Ph 7.2 to 7.4 and much less growth of B. pertussis, as indicated by a smaller number of colonies showing the lighter areas on the medium.

The next attempt was to find the limits of acidity favorable for the growth of B. pertussis. A series of careful preliminary tests was made to determine the Ph values of the mediums after the addition of different amounts of various acids. It was thought best to use organic acids for this purpose. Small amounts of acetic, lactic and tartaric acids were added to Bordet-Gengou and to potato-glycerol-veal agar in quantities sufficient to make the final dilutions from 1 : 2,500 to 1 : 100. The mediums were then tested as to their reaction.* These tests were made before the addition of blood, and the reaction may, therefore, have been changed slightly by the blood. On the advice of C. E. Ekroth, potassium oxalate was also tried. Though in itself it is neutral, the medium to which it is added becomes more acid. The results of this test as to acidity are shown in table 4.

TABLE 4.

REACTIONS OF MEDIUMS ADJUSTED TO DIFFERENT H-ION CONCENTRATIONS BY THE ADDITION OF VARIOUS ORGANIC ACIDS OR OF POTASSIUM OXALATE

Medium	Acid	Final Dilution 1:2,000 Ph	Final Dilution 1:1,000 **Ph**	Final Dilution 1:500 Ph	Final Dilution 1:100 Ph
Bordet Gengou	Lactic	4.4	4.1	3.7	3.2
Glycerol-potato-veal agar	Lactic	5.4	5.0	4.3	3.6
Bordet Gengou	Acetic	4.45	4.4	4.1	3.7
Glycerol-potato-veal agar	Acetic	5.3	5.0	4.4	4.1
Bordet Gengou	Tartaric	4.2	3.9	3.6	3.2
Glycerol-potato-veal agar	Tartaric	5.5	4.4	4.3	3.6
Bordet Gengou	Potas .oxalate*	4.4	4.5	4.5	4.4
Glycerol-potato-veal agar	Potas .oxalate*	4.4	4.5	4.4	4.5

* Only one sample marked "technical, neutral" gave us the reactions recorded in this table. The results in isolation continued to be satisfactory with the dilution of 1:2,000. Later tests of this sample, when added to the Bordet-Gengou medium, showed, however, a much less acid reaction (Ph 6.1 to 6.3) as did also other samples marked "tested purity" and "blue label reagent, neutral."

It is seen by this table that the same dilutions of the three acids have a more acid effect on the Bordet-Gengou medium than on potato-glycerol-veal agar. This is probably because the Bordet-Gengou medium contains no meat and has therefore practically no buffers.

Thus, while the reaction of potato-glycerol-veal agar after addition of each of the 3 acids in 1 : 2,000 dilution is from Ph 5.5 to 5.2, the reaction of Bordet-Gengou medium in the same dilution is from Ph 4.4 to 4.2. In a dilution of 1 : 1,000, the reaction of potato-glycerol-veal agar is from Ph 5 to 4.4; that of Bordet-Gengou is from 4.4 to 3.9. In 1 :500 dilution, the reaction of potato-glycerol-veal agar is from Ph 4.4 to 4.3; that of Bordet Gengou from 4.4 to 3.6. In 1 : 100 dilution, the reaction of potato-glycerol-veal agar is from Ph 4.1 to 3.6, while that of Bordet-Gengou is from 3.7 to 3.2. Potassium oxalate, on the other hand, seems to act on both mediums alike, and the reaction is the same (Ph 4.5 to 4.4) in all 4 dilutions.

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*The determinations were made by Mr. A. M. Last, chemist in the Division of Media. The colorimetric method was used.

To prepare these mediums it was found necessary to add the acids in suitable dilution to the medium after sterilization, just before addition of the blood. If the acids are added to the mediums before the autoclaving, the agar tends to lose its solidifying property. This is true especially when the stronger dilutions of acid are used. Agar containing dilutions of acid as high as 1:2,500 or 1:3,000 can be sterilized with little effect on its firmness. Since 1922 all sterilization of Bordet-Gengou medium has been carried out in the Arnold sterilizer for one hour on each of three successive days.

In making tests as to the limits of acidity favorable for the growth of B. pertussis, we confined ourselves entirely to the Bordet-Gengou medium, for reasons which will be given later.

Table 5 shows the limits of growth of pure cultures of B. pertussis on Bordet-Gengou medium with the different acids and potassium oxalate. The results as summarized are as follows:

Acetic Acid.—Growth is good on Bordet-Gengou medium with the addition of this acid in 1:2,000 dilution (Ph 4.45); growth also good in 1:1,000 dilution (Ph 4.4); growth is poor in 1:500 dilution (Ph 4.1); no growth occurred at all with 1:100 dilution (Ph 3.7).

TABLE 5

FORTY-EIGHT HOUR GROWTH OF B. PERTUSSIS ON BORDET-GENGOU MEDIUM ADJUSTED TO VARIOUS H-ION CONCENTRATIONS WITH DIFFERENT DILUTIONS OF VARIOUS ORGANIC ACIDS OR OF POTASSIUM OXALATE

Acid	Final Dilution 1:2,000 Ph	Growth	Final Dilution 1:1,000 Ph	Growth	Final Dilution 1:500 Ph	Growth	Final Dilution 1:100 Ph	Growth	Control Acid Added Without Blood
Acetic	4.45	++	4.4	++	4.1	±	3.7	—	—
Lactic	4.4	++	4.1	++	3.7	+	3.2	—	—
Tartaric	4.2	++	3.9	++	3.6	+	3.2	—	—
Potassium oxalate..	4.4	++	4.5	++	4.4	++	4.5	++	—

Lactic Acid.—Growth is good with 1:2,000 dilution (Ph 4.4); also good with 1:1,000 (Ph 4.1); fairly good with 1:500 dilution (Ph 3.7); no growth with 1:100 dilution (Ph 3).

Tartaric Acid.—Growth is good with 1:2,000 dilution (Ph 4.2); also good with 1:1,000 (Ph 3.9); fairly good in 1:500 dilution (Ph 3.6); no growth in 1:100 dilution (Ph 3.2).

With potassium oxalate, the growth is good with all 4 dilutions, namely, from 1:2,000 to 1:100 dilution. The primary dilution of potassium oxalate was made by adding 1 gm. to 10 cc. of distilled water, which made a 1:10 dilution. The same proportion was observed with tartaric acid.

With pure cultures of B. pertussis, the growth on a medium to which acid had been added was, as a rule, good as long as the blood in the medium remained bright red. This was usually the case with the mediums to which acids in dilutions of 1:2,000 or 1:1,000 were added. When the dilutions were 1:500 and lower, the medium turned brown and little or no growth of B. pertussis occurred. With potassium oxalate the medium remained red with all 4 dilutions.

ISOLATION OF B. PERTUSSIS IN A SECOND SERIES OF CASES ON AN ACID MEDIUM

A second series of isolations was undertaken to test the worth of the following mediums for isolation purposes: Potato-glycerol-veal agar plus blood, chocolate agar, Shiga medium and Bordet-Gengou medium.

Potato-glycerol-veal agar plus blood, on which B. pertussis in pure culture grows so luxuriantly immediately after isolation, was found not suitable for the actual isolation of the organism. The plates were invariably overgrown, because this medium is too favorable for the growth of the associated organisms of the mouth and nasopharynx.

Chocolate-veal agar had been used first by the writer in 1912-1913 in work on influenza, and later for pertussis; it was then referred to as "coagulated blood veal agar." This medium is not suitable for isolation because B. pertussis grows in it only after preliminary cultivation on Bordet-Gengou for a number of weeks, and the organism must become accustomed to the modified blood. After that, the growth becomes luxuriant.

The Shiga medium as described is not suitable because it also is too favorable for the growth of B. influenzae and the other organisms usually present in the sputum of pertussis patients.

Bordet-Gengou medium, which is made without meat or peptone, is less favorable for the growth of the associated organisms of the mouth and nasopharynx. This medium (of a reaction PH 6.1 to 5.0) was the best for purposes of isolation and it was, therefore, the medium selected for our further tests.

The reaction of the mediums for the second series of isolations were as follows:

1. Bordet-Gengou adjusted with normal NaOH to PH 7.2 to 7.4 (used exclusively in the first series of isolations).
2. Bordet-Gengou unadjusted, PH 5.8 to 6.1.
3. Bordet-Gengou adjusted with acetic or lactic acids to PH 5.
4. Bordet-Gengou with acetic, lactic, tartaric acids in 1:2,000 and 1:1,000 from PH 4.5 to 3.9.
5. Bordet-Gengou with potassium oxalate in 1:2,000 and 1:1,000 dilutions.
6. Chocolate-veal agar, PH 7.2 to 7.4.

Of the modifications described under 4, only Bordet-Gengou with lactic acid added to 1:2,000 dilution was retained. As mentioned before, B. pertussis grows well in pure culture with each of the 3 acids added in dilutions of 1:1,000 to 1:2,000. This is not true, however, when the same mediums are used for purposes of isolation. It was found that when the acids were added in the proportion of 1:1,000 dilution, all of the plates turned brown the next day and could not be used. When the acids were added in 1:2,000 dilution, the results were variable. In this dilution, the lactic acid proved better than either the acetic or tartaric acids in the same dilution. The most constant results were obtained with agars containing acetic and lactic acids, PH 5 (1:2,500 dilutions); with potassium oxalate 1:2,000 and occasionally in 1:1,000 dilutions.

Table 6 shows the superiority of the acid mediums over the medium with a reaction of PH 7.2 to 7.4. With the Bordet-Gengou medium of the latter reaction in the first series of cases, we succeeded, as stated, in isolating 8 cultures from a total of 35 cases, over 22.8%. In contrast to this figure, our second series of cases tested at the same stages of the disease with the same medium, but of acid reaction, showed over 43.5% isolations (10 in 23 cases).

TABLE 6.

ISOLATION OF B. PERTUSSIS ON ACID BORDET-GENGOU MEDIUM REACTION PH 6.1 TO 4.4

Second Series of Twenty-Six Cases

Number of Cases	Week of Whoop	Number of Isolations	Percentage of Isolations
7	1	5	over 71.4
7	2	3	about 43.0
6	3	1	about 17.0
3	4	1	over 33.0
3	5	0	

In the second series, there were really 26 cases including the three in the fifth week of the whoop. Of the 26 cases, 24 came from the home for children. In the majority of cases obtained from the home for children, there were 2 unfavorable factors: The first was the early age of the children, from 2 to 4 years, at which period sputum is obtained with difficulty; the second was that the nature of the disease was not recognized until the symptoms were more or less advanced and past the catarrhal stage. Under more favorable conditions than these, a still higher percentage of isolations with the acid medium could be expected.

Table 7 gives still further proof of the advantage of an acid reaction. Thus with Bordet-Gengou medium, reaction PH 7.2 to 7.4, only 4 of 23 cases gave cultures of B. pertussis. With the unadjusted Bordet-Gengou (PH 6.1 to 5.8) 7 of 23; with the Bordet-Gengou adjusted to PH 5 with acetic acid, 10 cultures were obtained. Bordet-Gengou adjusted with potassium oxalate in 1:2,000 dilution (reaction? see note to table 4), 10 positive cultures from 23 cases up to the fourth week of the whoop. As seen in table 6, no results were obtained from any cases in the fifth week of the whoop.

TABLE 7.

COMPARATIVE VALUES OF DIFFERENT REACTIONS OF BORDET-GENGOU MEDIUM FOR ISOLATION OF B. PERTUSSIS IN DIFFERENT STAGES OF THE DISEASE

Number of Cases	Week of Whoop	Reaction Adjusted with Normal Sodium Hydroxid to Ph 7.2-7.4	Reaction Unadjusted Ph 5.8-6.0	Reaction Adjusted with Acetic Acid to Ph 5.0 (Dilution 1:2.500)	Reaction Adjusted with Lactic Acid to Ph. 4.4 (Dilution 1:2,000)	Reaction Adjusted with Potassium Oxalate to Ph. 4.5 (Dilution 1:2,000)	Total Number of Isolations
7	1	3	4	5	4	5	5
7	2	1	2	3	2	3	3
6	3	0	1	1	0	1	1
3	4	0	0	1	0	1	1
3	5	0	0	0	0	0	0
Total 23	...	4	7	10	6	10	10

One objection to the acid reaction in the Bordet-Gengou medium is the fact that it also favorable for the growth of gram-negative cocci. This difficulty is more than compensated for by the control of the growth of B. influenzae and other organisms.

In order to ascertain whether B. pertussis could be isolated from conditions other than whooping cough, control tests were made, at the suggestion of Dr. W. H. Park. Fifteen sputums from children ill with scarlet fever and measles and various other infections, also three from adults ill with scarlet fever, were tested with the acid Bordet-Gengou medium. No B. pertussis was isolated in any case.

In further attempts to isolate B. pertussis from cases other than whooping cough, cultures were taken from 30 cases of croup, 50 cases of measles, 40 cases of scarlet fever (all patients in Willard Parker Hospital) and from a number of nurses and laboratory workers suffering from colds. In no instance was B. pertussis isolated; B. influenzae, however, was found in a majority of these cases, together with gram negative cocci, green streptococci and streptococcus hemolyticus.

In addition to the proportion of isolations, we were interested in ascertaining the length of time during which B. pertussis could be obtained from a given case.

Attempts were made in a number of cases to obtain repeated cultures; the sputum was obtained in 2 cases 3 times, and in 1 case 4 times. Positive results were obtained in 2 successive cultures from 2 cases only. The third cultures from the same cases were negative. This work is still in progress.[1]

In Copenhagen, according to Chievitz and Meyer, the children are readmitted to school after the fifth week of the disease after the cultures have proved negative twice, in spite of the fact that the children still continued to whoop and cough. The experiments of these two workers in this direction have convinced them that whooping cough is not infectious after the fifth or sixth week of the disease.

An attempt was made in 2 cases to use the medium mentioned by Inaba, but on account of the meager description given by this writer, we did not know whether we were using the same medium or not. We omitted from the unadjusted Bordet-Gengou medium the glycerol and potato extract. It is evident that such a medium would be even less favorable for the associated organisms than the Bordet-Gengou medium. On the other hand, it may lack sufficient nourishment for the development of B. pertussis. This is suggested by the fact that though we obtained positive results on this medium, the colonies were less numerous than on the other mediums used, and on all positive results were given with material from these 2 cases.

The Chievitz and Meyer droplet method mentioned before, was used in six cases of the second series with negative results. These cases were in the second week of the whoop. However, many colonies of B. influenzae appeared on chocolate Avery plates from the same cases by the droplet method.

SUBSEQUENT GROWTH OF B. PERTUSSIS

After isolation, this organism continues to grow well on Bordet-Gengou medium, especially on the unadjusted PH 5.8 to 6.1. It grows best on unadjusted potato-glycerol-veal agar (PH 5.8 to 6.1) plus blood. This medium should be excellent for the cultivation of large quantities of B.

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[1] We have not yet been able to find the pertussis bacillus after the fourth week of the whoop.

pertussis and for the making of antigens and vaccines. The medium which is in use at present in our laboratory for this purpose is chocolate-veal agar. Although B. pertussis grows luxuriantly on this medium, it does not do so until many weeks after isolation, as mentioned before; while on potato-glycerol-veal agar plus blood it grows at once.

B. pertussis grows just after isolation also on Shiga's medium but not as luxuriantly as on the potato-glycerol-veal agar plus blood or on chocolate veal agar, after preliminary cultivation.

SUMMARY

It was found that a definitely acid reaction in a suitable medium is favorable for the isolation and growth of B. pertussis. Such a reaction is especially valuable because it inhibits the growth of B. influenzae and other organisms found in the sputum of patients with pertussis.

The most favorable point of acidity for isolation was found to be at PH 5.

The limits of acidity favorable to the growth of B. pertussis are PH 6.1 to 4.4.

The unadjusted Bordet-Gengou medium for the cultivation of B. pertussis is usually of the reaction PH 5.8 to 6.1. If a more acid reaction is desired, this can be obtained by adding one of several organic acids to a medium, preferably after the latter has been sterilized and before the addition of the blood.

B. pertussis grows immediately after isolation most luxuriantly on potato-glycerol-veal agar unadjusted (PH 5.8 to 6.1) plus blood in proportion of 1:3 or 1:4 added at a temperature of 45 C.; but this medium is too favorable for the growth of various other organisms found in the sputum and is therefore not suitable for the isolation of B. pertussis.

On the other hand, the Bordet-Gengou medium, which contains neither meat nor peptone, is less favorable for the growth of the associated organisms of the mouth and nasopharynx. It was found, therefore, that this medium after adjustment to a suitable acid reaction (PH 5) was the best for the isolation of B. pertussis.

NOTE: Since the publication of the above, twenty more strains of the Bordet-Gengou bacillus have been isolated from sporadic cases of whooping cough, using our modified acid medium. According to the agglutinin absorption test all thirty-eight strains isolated by the writer belong to type B (the type to which the original Bordet-Gengou strain belongs). The percentage of isolation has been very high—from seventy-five to eighty-five per cent.—in the first week of the whoop.

I am indebted to Miss Mildred S. Hollinshead for her assistance in this work.

For bibliography see the original article.

THE EXISTENCE OF MORE THAN ONE IMMUNOLOGIC TYPE OF B. PERTUSSIS

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CHARLES KRUMWIEDE; LUCY MISHULOW, AND CAROLYN OLDENBUSCH

In an investigation of the antigenic properties of pertussis vaccine, it became necessary to isolate a new strain of B. pertussis for comparison with older stock strains. Several strains, therefore, were isolated having all the typical morphologic and cultural characteristics of the Bordet-Gengou bacillus. These strains were tested agglutinatively with the serum of a rabbit which had been inoculated with the old stock strain utilized in the vaccine experiments. This serum agglutinated the freshly isolated strains nearly as well as the homologous serum strain. Rabbits were then injected with a suspension of one of the newly isolated strains. This serum to our surprise caused only slight agglutination (1:100) of the old stock strain, whereas the other freshly isolated strains were agglutinated to practically the same degree as the serum strain.

These observations led to a more detailed study of a series of strains of B. pertussis, utilizing agglutination and agglutinin-absorption for differentiation. From various sources we collected a series of stock strains among which was a strain with a history of having been obtained from Bordet. Fresh strains were obtained from 17 cases. For most of these strains we are indebted to Dr. Olga R. Povitzky, who isolated them while working on the improvement of mediums for the isolation and cultivation of B. pertussis.

As may be seen from tables 1, 2 and 3, the strains tested by agglutination and agglutinin-absorption fall into 2 groups, A and B. The tendency of group B is to cross agglutinate with serums of group A., also to absorb a large proportion of the specific agglutinins. Group A. strains, on the other hand, practically do not agglutinate with serums of group B and show little tendency to reduce the specific agglutinins.

TABLE 1

PRELIMINARY GROUPING BASED ON THE RESULTS OBTAINED WITH A LARGE ABSORBING DOSE 1:10

Strains Tested	Serum 253			Serum Bordet P. D.		
	Agglutination			Agglutination		
	Before Absorption	After Absorption		Before Absorption	After Absorption	
		Absorbing Strain	Serum Strain		Absorbing Strain	Serum Strain
Group A						
253	2,000++	0	0	0	0	1,000++
M 254, 1	1,500++	0	0	100++	0	2,000++
217 L	1,000++	—	—	0	0	1,000++
246 L	1,000++	—	—	0	0	1,000++
247 L	500++	—	—	0	0	1,000++
248	1,500++	0	0	0	0	1,000++
250 L	1,000++	—	—	0	0	1,000++
251	1,500++	0	0	0	0	1,000++
252 M	1,500++	—	—	0	0	2,000++
Group B						
Bordet P. D.	1,000++	0	200++	2,000++	0	0
0590 P D	1,000++	0	200++	1,000++	0	0
98 P D*	800++	0	0	1,000++	0	0
110 P D	800++	0	100++	1,000++	0	0
163 P D	800++	0	400++	1,000++	0	0
248 P D	500++	0	200++	1,000++	0	0
248 M	800++	0	400++	1,000++	0	0
M 254,4	1,000++	0	150++	1,000++	0	0
M 255,4	800++	0	400++	1,000++	0	0
M 256,11	800++	0	200++	1,000++	0	0
P 257,5	1,000++	0	100++	1,000++	0	0

* In dose of 1:80 no agglutination of absorbing strain in dilution of 1:25, but marked agglutination of serum strain at 1:400.

In this and the following tables, "Bordet" is a strain obtained from Bordet in 1911.

The numbers below 254 refer to strains isolated in this laboratory 1913-14; letters after the numbers designate laboratories from which the strains, originally sent out from this laboratory, were obtained. Strain 0590 was isolated about 1911.

Strains 254 and higher were isolated between February, 1921, and April, 1922, by Povitzky (P) and Mishulow (M); the number after the comma refers to individual fishings.

In all tests 1:100 is the lowest dilution used in the direct agglutination tests; 1:25 is the lowest dilution used after absorption, and in all cases in which there was no agglutination in this dilution, higher dilutions were without effect also; 1:200++ etc., indicates highest dilution giving marked but partial agglutination.

+++, complete agglutination; ++, marked but partial agglutination; +, slight agglutination; 0, no agglutination; —, not tested.

TABLE 2

CONFIRMATORY RESULTS OBTAINED WITH ANOTHER SERUM, USING A LARGE ABSORBING DOSE 1: 10

Strains Tested	Serum 251: Agglutination		
	Before Absorption	After Absorption	
		Absorbing Strain	Serum Strain
251	4,000++	0	0
253	4,000++	0	0
M 254,1	6,000++	0	0
0590 P D	1,000++	0	3,000++
98 P D	1,000++	0	3,000++
110 P D	2,000++	0	3,000++
163 P D	1,000++	0	3,000++
M 254,4	600++	0	1,500++
M 255,4	2,000++	0	100++
M 256,11	1,000++	0	1,500++

Other confirmatory tests were made with serum M 255,4, testing 16 strains, and with serum M 254,4, testing 11 strains. The results with these serums were so closely like those obtained with serum Bordet (table 1) that there seems to be no reason for publishing the tabulation of the results.

TABLE 3

ABSORPTION OF SERUM "BORDET" GROUP B. BY STRAINS OF GROUP B., ABSORBING DOSE, 1: 200

Strains Tested	Agglutination Before Absorption	Agglutination After Absorption	
		Absorbing Strain	Serum Strain
Bordet P D	2,000+	0	0
0590 P D	1,000++	0	0
98 P D	2,000+	0	0
110 P D	2,000+	0	0
163 P D	2,000+	0	0
248 P D	1,000++	0	0
248 M	1,000++	0	0
249 L	1,000+	0	0
M 254,3	1,000++	0	0
M 255,1	1,000+++	0	0
M 256,1	1,000++	0	0
P 257,5	2,000+	0	0
P 258,1	1,000++	0	0
P 259,1	2,000+	25+	0
P 260,1	2,000+	0	0
P 261,1	1,000++	0	0
P 262,8	2,000+	25++	25++
P 263,5	2,000+	25+	25+
P 264,4	2,000+	25+	25+

TABLE 4.

ABSORPTION OF SERUM 253 GROUP A BY STRAINS OF GROUP A. ABSORBING DOSE, 1: 200

Strains Tested	Agglutination Before Absorption	Agglutination After Absorption	
		Absorbing Strain	Serum Strain
253	2,000+	50+	50+
M 254,1	2,000+	26+++	50+
217 L	1,000++	0	0
246 L	1,000++	0	26+
247 L	400+++	0	60+
248	1,000+++	25++	26++
250 L	1,000+++	25++	25++
251	2,000+	25++	25++
252 M	1,000+++	25++	25++

As is noted in table I, both type A and type B were isolated from case 254. This led us, when possible, to make numerous fishings from the plates inoculated with the sputum from the cases of pertussis which came to us later. These fishings have been tested and grouped either by agglutination alone or by agglutination and agglutinin-absorption. The number of cases and fishings per case thus tested are noted in table 5. All the cases with the exception of 254 have yielded only strains of group B.

The grouping of all the strains studied may be summarized thus: Among the old stock strains, both types were encountered. In some instances, transplants of the same cultures carried by different laboratories have been

TABLE 5.

TABULAR SUMMARY OF STRAINS FROM INDIVIDUAL CASES

Cases	Number of Fishings Tested	Grouping			
		Group A		Group B	
		Classified on the Basis of Direct Agglutination Only	Classified on the Results of Agglutination and Absorption	Classified on the Basis of Direct Agglutination Only	Classified on the Results of Agglutination and Absorption
M 254	4	0	1	0	3
M 255	20	0	0	14	6
M 256	21	0	0	16	5
P 257	2	0	0	0	2
P 258	15	0	0	11	4
P 259	7	0	0	4	3
P 260	4	0	0	2	2
P 261	2	0	0	1	1
P 262	35	0	0	26	9
P 263	15	0	0	11	4
P 264	1	0	0	0	1
P 265	1	0	0	0	1
P 266 a+	1	0	0	0	1
P 266 b+	1	0	0	0	1
P 267	3	0	0	2	1
P 268 a+	3	0	0	2	1
P 268 b+	3	0	0	2	1
P 269	3	0	0	3	0
P 270	3	0	0	3	0

a+ and b+ = successive isolations from the same case with an interval of two weeks.

found to be different. This must be due either to mislabeling or to the fact that the original strains contained a mixture of the types. In one instance there was apparently a mixture in one of the stock cultures. From the recent cases, one case yielded both types, but all the other cases have yielded only type B. What interpretation is to be made of these results it is impossible to state. It may be that the different types may vary in their prevalence at different times; or it may be suggested that only one of the types is a true B. pertussis. This cannot be disproved, but the presumption is against this conclusion in view of the morphologic and cultural similarity as well as the agglutinative relationship. It may be argued that the differences are due to a change of serologic characteristics among some of the stock strains. This argument cannot be considered in view of the isolation of both types from a recent case (254).

In view of the report of Bordet and Sleeswyk that they could influence the antigenic characters of B. pertussis by the use of different mediums, all our serologic tests were carried out with bacilli cultivated on one type of medium, namely, chocolate agar. Bordet and Sleeswyk reported that if they immunized a horse or a rabbit with bacilli grown on a blood containing medium, such as the Bordet-Gengou medium, that the resulting agglutinins were active against bacilli grown on the same medium, but less active against bacilli grown on plain agar. The agglutinins could be completely absorbed from such a serum by bacilli grown on the Bordet-Gengou medium but not by the same strain grown on plain agar. If a horse or a rabbit were injected with bacilli grown on plain agar, the results were reversed. If the strain grown on plain agar were again cultivated on Bordet-Gengou medium, its agglutinative characteristics became the same as the strain continuously grown on a blood medium.

These results have never been confirmed to our knowledge. Povitzky found that horse injected with two strains grown on Bordet-Gengou yielded a serum during the earlier period of immunization which agglutinated bacilli grown on the same medium but not those grown on chocolate agar. After a longer period of immunization no such differences were noted. Working with rabbit serums she found no agglutinative or absorptive differences in regard to the mode of cultivation of the bacilli used.

We have made comparative tests with serums obtained from rabbits injected with B. pertussis grown on chocolate agar. Antigens were prepared from the bacilli grown for from 5 to 20 generations on plain agar, chocolate agar, Bordet-Gengou medium containing about 25% of unheated blood, and a medium studied by Povitzky. This medium was a potato-glycerol-veal agar which contained about 35% of unheated blood.

Table 6 is representative of the results obtained. With the older stock strains, no appreciable differences in agglutinative or absorptive characteristics were noted. With the more recently isolated strains, which, however, could not be grown on plain agar, considerable differences were observed. The antigens grown on chocolate agar (heated blood) agglutinated well and showed a high absorptive capacity. Those from mediums containing unheated blood (either Bordet-Gengou or Povitzky) agglutinated poorly or in some instances not at all and had a low absorptive capacity. A transfer for even one generation to chocolate medium resulted in a complete return to the higher level of agglutinability and absorptive capacity.

A serum from a rabbit injected with a recently isolated strain grown on the Bordet-Gengou medium was tested in the same way. This serum gave much better agglutination with bacilli grown on the chocolate medium.

TABLE 6

THE INFLUENCE OF THE MEDIUM ON AGGLUTINATION AND AGGLUTININ-ABSORPTION; SERUM BORDET, FROM RABBIT IMMUNIZED WITH BACILLI GROWN ON CHOCOLATE AGAR

Agglutination Before Absorption			Agglutination After Absorption				
			Absorbed By		Tested With		
Strain	Grown On	Titer	Strain	Grown On	Strain	Grown On	Titer
Bordet	Chocolate agar	3,000++	Bordet	Chocolate agar	Bordet	Chocolate agar	0
						Povitzky	0
						Bordet-Gengou ...	0
						Agar	0
Bordet	Povitzky	3,000+	Bordet	Povitsky	Bordet	Chocolate agar	0
						Povitzky	0
						Bordet-Gengou	0
						Agar	0
Bordet	Bordet-Gengou	3,000++	Bordet	Bordet-Gengou	Bordet	Chocolate agar	0
						Povitzky	0
						Bordet-Gengou	0
						Agar	0
Bordet	Agar	3,000+	Bordet	Agar	Bordet	Chocolate agar	0
						Povitzky	0
						Bordet-Gengou ...	0
						Agar	0
P 264.4	Chocolate agar	3,000++	P 264,4	Chocolate agar	P 264,4	Chocolate agar	0
						Povitzky	800+
						Bordet-Gengou ...	800+
P 264.4	Povitzky	1,600+	P 264,4	Povitzky	P 264,4	Chocolate agar	400+
						Povitzky	400+
						Bordet-Gengou ...	800+
P 264,4	Bordet-Gengou	3,000+	P 264,4	Bordet-Gengou	P 264,4	Chocolate agar	100+
						Povitzky	800+
						Bordet-Gengou	800+

When the serum was absorbed by bacilli grown on the chocolate medium, the agglutinins for the chocolate-agar-grown bacilli were removed; when absorbed by bacilli grown on the Bordet-Gengou medium, these agglutinins were only reduced. In neither instance, however, was there an appreciable reduction in the "agglutination" of the Bordet-Gengou antigen. These results are given in table 7. The fact that the absorption was not followed by a reduction in

TABLE 7.

THE INFLUENCE OF THE MEDIUM ON AGGLUTINATION AND AGGLUTININ-ABSORPTION; SERUM M 255 FROM RABBIT IMMUNIZED WITH BACILLI GROWN ON BORDET-GENGOU MEDIUM.

Agglutination Before Absorption			Agglutination After Absorption				
			Absorbed By		Tested With		
Strain	Grown On	Titer	Strain	Grown On	Strain	Grown On	Titer
M 255.4	Chocolate agar	2,000++	M 255,4	Chocolate agar	M 255,4	Chocolate agar...	0
						Bordet-Gengou ...	800+
M 255,4	Bordet-Gengou	1,000+	M 255,4	Bordet-Gengou	M 255,4	Chocolate agar....	50++
						Bordet-Gengou ..	800+
P 270.3	Chocolate agar	2,000+	P 270,3	Chocolate agar	P 270,3	Chocolate agar....	0
						Bordet-Gengou ...	400+
					M 255,4	Chocolate agar....	0
						Bordet-Gengou ...	800+
P 270.3	Bordet-Gengou	800+	P 270,3	Bordet-Gengou	P 270,3	Chocolate agar....	50++
						Bordet-Gengou ..	400+
					M 255,4	Chocolate agar....	0
						Bordet-Gengou ...	1,000+

the degree of clumping of the Bordet-Gengou antigen. raised the question whether the flocculation with this type of antigen was due to specific agglutination.

The two types of antigens of the freshly isolated strain were tested, therefore, with 2 normal rabbit serums; also with 2 homologous serums, one from a rabbit injected with bacilli grown on the Bordet-Gengou medium, the other from a rabbit injected with bacilli grown on chocolate agar. Both the immune serums agglutinated the chocolate-agar antigens, whereas the normal serums did not. All the serums, including the normal, "agglutinated" the antigens prepared on Bordet-Gengou medium (horse blood) equally well. Antigens grown on a Bordet-Gengou medium containing rabbit blood were not "agglutinated" by the normal serums but showed a slight reaction with the immune serums. At first glance this would seem to indicate that the clumping of the Bordet-Gengou (horse blood) antigen was a precipitation reaction between rabbit and horse serum. Antigens of old stock strains, however, did not show the same results. As has been noted in the foregoing, the agglutinability and absorptive capacity of the older stock strains were not influenced by the medium on which they were grown. Why only the freshly isolated strains should be influenced by the medium and why only the antigens of such strains grown on unheated blood mediums should give nonspecific clumping, remains to be ascertained. These results again emphasize the dangers of employing antigens obtained from mediums containing unheated blood or serum. The recent report of Kelly, who found similarly bizarre reactions in studying the precipitin reaction in gonorrheal infections, is of special interest in this connection.

The results we have obtained do not in any way confirm the findings of Bordet. The observation of Povitzky that the antigens of recently isolated strains grown on the Bordet-Gengou medium were agglutinated much better during the earlier period of immunization is apparently explained by our observations; that is, the reaction was due to the horse serum without regard to its content of specific agglutinins.

Summary

A series of strains having the typical morphologic and cultural characteristics of B. pertussis have been studied by means of agglutination and agglutinin-absorption. These tests have demonstrated that the cultures studied fell into two serologic groups. These groups are designated as A and B. Antiserums for group B agglutinate the strains of group B but agglutinate the strains of group A slightly or not at all. Group A serums, however, agglutinate not only the strains of group A, but also agglutinate the strains of group B to a considerable extent. The absorption of group B serums by strains of group A does not appreciably reduce the content of agglutinins for strains of group B. The absorption, however, of group A serums by strains of group B results in a reduction in the agglutinins for strains of group A. The serologic differences are therefore sharply defined in one direction, but group relationship is shown in the reverse direction. The strains within each group are alike as far as is indicated by the absorptions of serums for different members within the same group.

The antigens for these tests were all prepared from the same medium; the serologic differences cannot be attributed therefore, to the influence of different mediums. The growth on blood mediums influences the ag-

glutinative characteristics of freshly isolated strains of B. pertussis but we have been unable to confirm Bordet's observations on this point.

The existence of two groups of B. pertussis is of immediate interest because of their possible bearing on the use of pertussis vaccine.

With the exception of one case of whooping cough which showed the presence of both types, all the recent cases have yielded only strains of group B.

For bibliography see the original article.

PERTUSSIS VACCINATION AS A PROPHYLACTIC MEASURE

Anna I. Von Sholly

During 1920-1921 the children under 10 years of age in 24 institutions were inoculated as follows:—1823 children received pertussis bacillus (hemophilus pertussis) type A saline solution, 3 strains varying from 8 days to 6 months old, dose 10 billion divided into 3 doses; 509 children received glycerole vaccine, pertussis A and B, 10 billion divided into 3 doses; 42 children received saline solution, type B. pertussis (original Bordet-Gengou bacillus). This made a total of 2374 children. At the same time 1971 children were inoculated with a vaccine composed of 6 strains of influenza bacilli, or with type 1, 2 and 3 pneumococcus vaccine—as controls.

Results:—Within the two following years whooping cough appeared in 11 institutions from two months to nineteen months after vaccination, as follows:—29 children who had been inoculated with type A pertussis vaccine developed pertussis, that is, 2.3% in the 11 institutions; 4 children who had been inoculated with glycerole A and B types of pertussis bacilli developed the disease, that is, 1.2% in the 11 institutions; 17 children who had been inoculated with pneumococcus or influenza vaccine developed pertussis, that is, 1.2% of the vaccinated control group in these 11 institutions. Among the unvaccinated controls, that is, children who were admitted subsequent to the vaccinations, 73 children or 6.7% of this group developed the disease.

In late 1922, two years after the first inoculations, the children of twelve of the same institutions were inoculated as follows:—of the children then in the institutions, those who previously had received pertussis vaccine were re-inoculated with the same, and those used as controls were continued as such. The new children were inoculated with pertussis vaccine and control vaccine alternately. Instead of type A pertussis vaccine, type B—the original Bordet-Gengou type—was used in 21 billion dosage divided into 4 doses. There were 605 re-inoculated children.

In all, 887 children received pertussis vaccine type B and 756 children received pneumococcus vaccine as controls, making a total of 1643 vaccinated children.

The results up to June 1st, 1924 are as follows:—whooping cough has appeared in 4 institutions among 4 children who received pertussis type B or 1.1% of this group in the 4 institutions; 9 children who received pneumococcus vaccine or 3.17% of this group of vaccinated controls; 39 children who were not vaccinated (new admissions) or 6.09% of this group.

In this investigation pertussis vaccine type B seems more favorable as a prophylactic agent than type A used in the first experiment.

POTENCY OF STORED PERTUSSIS VACCINES

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Lucy Mishulow, Carolyn Oldenbusch and Marie Scholl

The reports of the value of pertussis vaccine as a prophylactic and curative measure are contradictory. Some are favorable, whereas others indicate practically negative results. The claim has been advanced that freshly prepared pertussis vaccines are more antigenic than stored vaccines. Thus Huenikens reported his results on a series of children whom he immunized with pertussis vaccines. He treated one group with vaccines that were less than one week old; and a second group with vaccines that had been stored for varying periods. Two weeks after the last injection he tested their serums for complement-fixing antibodies. Thus measuring immunity by the stimulation of complement-fixing antibodies, he found that vaccines that had been stored for 2 to 3 months immunized in only about 12.5% of the number tested. On the other hand, freshly prepared vaccines employed within a few days of preparation immunized in about 94%. But the freshly prepared vaccines that he used and those that he designated as stored, were not the same preparations. He used commercial vaccines in his tests of stored vaccines and compared their potency with those that he prepared himself and used soon after preparation. In our experience with bacterial vaccines we find that there may be wide variations in the potency of different lots of vaccine prepared at different times using exactly the same technic. Therefore, in order to compare the potency of fresh and stored vaccines the same preparation should have been employed throughout the test. Others reported favorably on the use of fresh pertussis vaccines as compared with stored vaccines basing their observations on clinical results (Freeman, Moody and others).

The continuous preparation of fresh vaccines on a large scale is laborious and time consuming. For this reason it is undesirable unless proven to be of greater efficacy than properly stored vaccines. The investigations, therefore, that we carried out have been to determine primarily whether the antibody response of laboratory animals injected with freshly prepared and with stored vaccines would yield any data corroborative of the above claims. The following questions concerning the best method for preparing potent pertussis vaccines were also investigated. Does a freshly isolated strain have greater antigenic value than a strain that has been under artificial cultivation for a number of years? Do vaccines that are grown on chocolate agar differ in antigenic value from vaccines that are grown on Bordet-Gengou medium? Have salt solution preparations the same keeping qualities as glycerol preparations? Does heating the vaccine have any effect on the potency of stored vaccine? Have preservatives any effect on the potency of stored vaccine?

Several lots of vaccines were prepared and tested as follows.

Lot 1.—A strain of pertussis that has been under cultivation for a number of years was grown on coagulated blood agar (chocolate agar) for 48 hours then the growth was divided into two parts.

Vaccine 1: The growth was scraped into sterile 0.85% salt solution, shaken by hand to break up clumps, and heated at 53° C. for one hour. It was then tested for sterility and enough phenol added to make 0.5% of the bulk. The concentration was determined by Wright's method and the vaccine was diluted with 0.3% phenol in 0.85% saline to contain 1,000 million bacteria per cc.

Vaccine 2. The growth was scraped into sterile 66⅔% glycerol in salt solution and shaken in a mechanical shaker for one-half hour to break up clumps. It was standardized by Wright's technic and further diluted with 66⅔% glycerol to a concentration of 40,000 million bacteria per cc. Phenol was added to make 0.3% of the bulk. Just before using, the vaccine was diluted with sterile physiologic salt solution (which had 0.3% phenol) to contain 1,000 million bacterial per cc.

Lot 2.—Vaccines 3 and 4 were prepared in the same manner as lot 1, with salt solution and glycerol respectively, only a freshly isolated strain was used instead of the old stock strain. The object was to determine, if possible, whether the length of time of artificial cultivation of the strain has any influence on the antigenic value of the vaccine.

Lot 3.—In this preparation a mixture of three old stock strains was used, all of them serologically identical (type A). They were grown on Bordet-Gengou medium for 48 hours and the growth scraped into sterile distilled water. The clumps were broken up by shaking by hand and after the concentration was determined by Wright's method, the vaccine was divided into two parts; one part was heated at 53° C. for one hour. Then an equal volume of sterile 1.7% salt solution was added to both the heated and unheated vaccines thus making the concentration of NaCl in the vaccine 0.85%. Each vaccine was again divided into two parts and phenol was added to alternate lots as follows: vaccine 5, unheated, no phenol added; vaccine 6, unheated, 0.5% phenol; vaccine 7, heated, no phenol added, and vaccine 8, heated, 0.5% phenol.

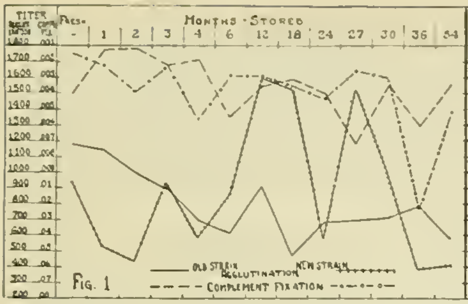
The object of this vaccine was to determine whether the medium on which the vaccine was grown and the heating of the vaccine in the process of preparation had any influence on the potency of the vaccine. Also, if there is any deterioration during the time of storage, whether phenol has any influence on the rate of deterioration.

Lot 1 (vaccines 1 and 2) and lot 2 (vaccines 3 and 4) were tested for potency as soon as they were found to be sterile, usually within two days, and the bulk was stored in the icebox at 8° C. to 10° C. and tested at intervals for four and one-half years (figs. 1 and 2). Due to pressure of other work at the time, vaccine lot 3 was not tested until after it was stored for 10 months, but after that it was tested at intervals until it was five and one-half years old (fig. 3).

Fresh vaccines were prepared of the strains used in the stored vaccines, and tested at the same time as the stored vaccines in order to control, more or less, the response of each lot of rabbits to immunization (figs. 4 and 5).

Method of Immunization.—A series of 4 to 6 rabbits was used for each of the preparations and each of the rabbits received 250 million bacteria intravenously for the first dose, followed by two more doses of 500 million each at intervals of 4 or 5 days. The rabbits were bled on the seventh day after the last injection and their serums were tested individually for agglutinins, and complement-fixing antibodies. Also the serum of each rabbit was tested before immunization to rule out nonspecific reactions.

Methods of Testing.—Agglutination: After some preliminary tests we found that antigens prepared of the old stock strain and of the freshly isolated strain gave practically the same titer of agglutination with



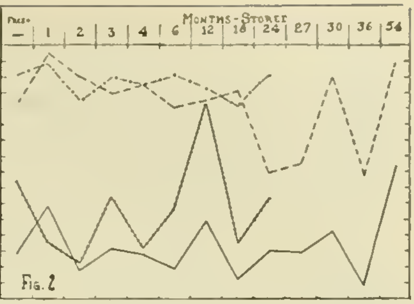


Fig. 1.—Complement-fixing and agglutinating antibody response of rabbits immunized with salt solution vaccines. Growth on chocolate agar; stored and tested at intervals.

Fig. 2.—Response to immunization with glycerol vaccine, similarly prepared and stored.

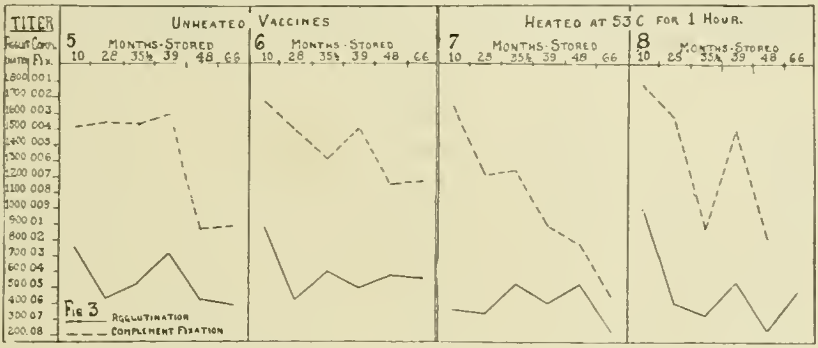
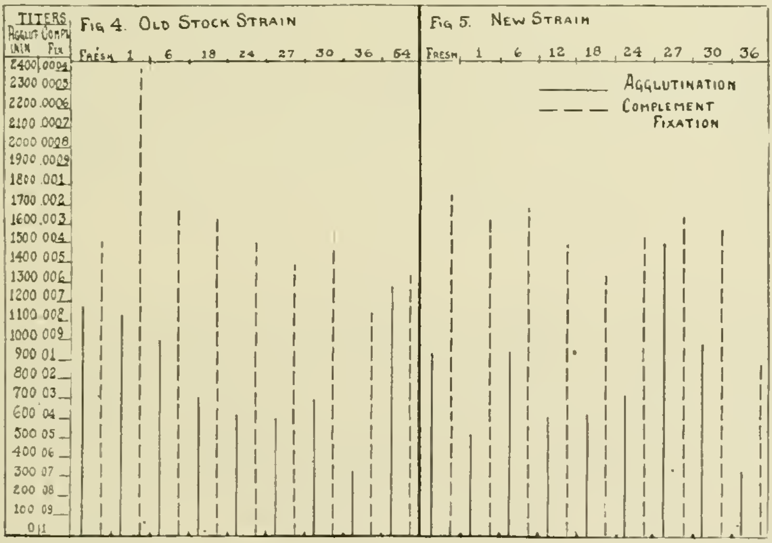


Fig. 3.—Response to immunization with salt solution vaccine. Growth on Bordet-Gengou medium; storage at 8°—10° C. Preparations 5 and 7 unheated and heated, respectively, contained no preservative, and 6 and 8 contained 0.3% phenol.



Figs. 4 and 5.—Variations in response of rabbits to immunization with freshly prepared salt solution vaccines. Growth on chocolate agar. These recorded responses were to freshly prepared vaccines, and were used as controls, respectively, for vaccines stored from 1 to 54 months as indicated by the figures of the heading.

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all the serums tested. Antigens of the old strain gave somewhat more uniform results and were therefore selected for testing all the preparations. A 24 to 48-hour growth on chocolate agar was scraped into a solution of formaldehyde (0.1% formalin in physiologic salt solution) and shaken for a few minutes to break up clumps. It was filtered through cotton and diluted with the formaldehyde-salt solution to the desired concentration, which was determined by preliminary tests. The antigen can be kept in the icebox for a year or more unless it is contaminated.

Serums of normal rabbits frequently gave agglutination in dilutions below 1:50; but in only one instance was there more than a trace of agglutination in a 1:50 dilution. Therefore to rule out nonspecific agglutination, the lowest dilution of the immune serums in our tests was 1 : 100. The serums were diluted 1:10, 1:20, etc. with physiologic salt solution. Then to 0.1 c.c. of each dilution of the serum, 0.9 c.c. of the antigen was added and the tests were incubated at 55° C. overnight. The highest dilution of the serum that showed strong agglutination was taken as the titer of that serum.

Complement Fixation: A 24 to 48-hour growth on chocolate agar was scraped into the formaldehyde solution (0.1% formalin in physiologic salt solution). The clumps were broken up by shaking and the suspension filtered through a thin layer of absorbent cotton and centrifuged. The sediment was resuspended in the formaldehyde-salt solution and again centrifugated. The sediment was then suspended in a formaldehyde-salt solution (formalin 0.5%) and shaken for 7 hours in a mechanical shaker. The antigen was titrated with rabbit immune serums and so standardized that four times the amount used in the tests was not anticomplementary and one-fourth of the test dose gave complete fixation with four units of the immune serum.

For the hemolytic amboceptor rabbits were immunized with washed red blood cells of sheep and their serums used as hemolytic amboceptor.

Guinea-pig serum was used for complement. Not all guinea-pig serums could be used in the tests as some gave nonspecific fixation and others gave weak or no fixation with positive serums. It was therefore necessary to test the serums of each guinea-pig before it could be used in the tests. Four to six guinea-pigs were bled on the morning preceding the day of the tests. As soon as the blood was clotted and some serum could be obtained by centrifugating, each serum was tested for hemolysis and complement-fixation. Those that were found suitable were pooled and stored until the following morning at a temperature slightly above freezing. The pooled serum was then titrated for the hemolytic unit, and was so diluted that 0.1 c.c. contained two hemolytic units.

The serums before testing were inactivated at 56° C. for ½ hour. Each serum of the rabbits immunized with the vaccines was diluted 1:10 and 1:100 with physiologic salt solution and titrations for antibody content were made, using two units of complement and 0.1 c.c. of the standard antigen. The tests were incubated in a waterbath at 37° C. for one hour to allow fixation to take place and then 0.2 c.c. of sensitized sheep red blood cells were added and the tests reincubated for another hour. The hemolytic system was so adjusted that the controls of the serum without the antigen, also of the antigen without the serums, were completely hemolysed in 15 to 20 minutes. The additional incubation of the tests after the controls were hemolysed helped to further rule out weak or doubtful reactions as the unhemolysed blood cells would settle to the bottom of the tubes leaving the supernatant fluid clear in the tubes where fixation was complete. In charting the results, the smallest amount of the serums that gave complete fixation (++++) was taken as the titer of that serum.

In testing the serums of the rabbits immunized with vaccines lots 1 and 3 for complement-fixing antibodies, an antigen prepared of the old strain was used. The serums of the rabbits immunized with vaccines of the new strain were tested with both the old strain and the new strain. There was very little difference in the titer of the serums when tested with the two antigens and for the sake of uniformity the results with antigen from the old strain were taken for all the different lots of vaccines.

The serum of each rabbit was tested for complement-fixing antibodies before immunization. In no instance did we get strong fixation in .01 c.c. of the serums and only very rarely was there strong fixation in .02 c.c. Usually they were either completely negative or gave only partial fixation in .02 c.c.

We found considerable variation in the individual response of rabbits in each test. In order to obtain a fair estimate of the results, although each rabbit was tested individually, we used in charting the average response of each series of rabbits in each test (figs. 1, 2 and 3).

Discussion

The results of our investigations which extended over a period of five and one-half years and in which we tested several different lots of pertussis vaccines when freshly prepared and after storing, show that there was no measurable deterioration in their power of stimulating complement-fixing antibodies in rabbits. The vaccine of the old stock strain prepared in salt solution (vaccine 1) showed gradual slight deterioration of agglutinogenic power. But it is difficult to draw conclusions from that, as the salt solution vaccine of the newly isolated strain (vaccine 3) showed no such deterioration. The glycerol preparations of both the old stock strain and the newly isolated strain showed no deterioration in their power of stimulating agglutinins and complement-fixing antibodies in rabbits.

There was a marked fluctuation in the response of different lots of rabbits that were immunized with the various preparations as well as individual rabbits in each lot. But these fluctuations were similar in every respect to the fluctuations shown by rabbits when immunized with freshly prepared vaccines tested at different times (figs. 4 and 5). The fluctuations in the agglutinin response of rabbits to immunization with vaccine from the new strain were much greater than in those immunized with vaccine from the old strain; but on the whole they seem to be alike in their antigenic value.

The vaccines prepared of a mixture of three old stock strains and were grown on Bordet-Gengou medium, some of them heated and stored with and without preservatives were not tested until after they were 10 months old. Figure 3 shows that the unheated vaccine without preservative showed no deterioration until it was stored for 48 months. Then there was a decided drop in agglutinogenic power as well as in its power to stimulate complement-fixing antibodies. The same vaccine with phenol gave practically the same results. The heated vaccine without preservative showed a marked deterioration in its power to stimulate complement-fixing antibodies between 10 and 28 months storage, which continued decreasing during the entire period of testing (66 months) but showed practically no decrease in agglutinogenic power. The heated vaccine plus preservative, after an initial drop in both agglutinins and complement-fixing antibodies between 10 and 28 months storage showed no further deterioration, only the fluctuations due to the difference in response to immunization of different lots of rabbits.

The four preparations in this lot of vaccine (lot 3) were tested simultaneously; i. e., the rabbits were immunized simultaneously and their serums were tested on the same day with the same reagents. In this way the differences elicited can not be attributed to possible variations due to the reagents employed. The heated salt solution vaccine plus phenol was found to retain its potency much longer than the heated salt solution vaccine without phenol. The unheated salt solution vaccine with and without phenol retained its potency for a longer period than the heated salt solution vaccine without phenol; but not as long as the heated salt solution vaccine to which phenol was added.

Harvey and Iyengar in their experiments on pigeons found that agglutinin and complement-fixing antibody response run parallel with the protective power up to 10 months' storage. After that the protective value deteriorates, but the agglutinin and complement-fixing antigenic powers remain high for a much longer period. We could not investigate this claim, as the pertussis bacillus is not sufficiently virulent for laboratory animals to test the protective value of the vaccine. In regard to the production of agglutinin and complement-fixing antibody, however, our results may be summarized as follows:

Pertussis vaccines prepared in salt solution, heated at 53 C. for one hour, containing 0.3% phenol, were found to retain their power of stimulating the production of agglutinins and complement-fixing antibodies in rabbits for a period of from 4½ to 5½ years, when stored at 8° to 10° C. When stored without preservatives such heated, salt solution vaccines deteriorated more rapidly than either the heated vaccine plus preservative or the unheated vaccine. Unheated salt solution vaccine with and without preservatives differ very little from heated vaccines to which preservative was added, when tested after a comparatively short period of storage. On longer storage there was considerable deterioration.

Vaccines prepared of a newly isolated strain of pertussis showed no greater antigenic power than vaccines prepared of an old laboratory stock strain.

Glycerol preparations of both the old stock strain and the newly isolated strain showed no appreciable deterioration in their power of stimulating agglutinins and complement-fixing antibodies in rabbits for a period of 4½ years.

Vaccines grown on chocolate agar did not differ in their antigenic power from those that were grown on Bordet-Gengou medium.

CONCLUSIONS

If the antigenic value of pertussis vaccines can be measured by their power to stimulate specific antibodies, and if the response in human beings to immunization with the vaccines is similar to that in rabbits, properly stored vaccines remain antigenically potent for a considerable length of time. The claim that stored pertussis vaccines lose about 75% of their antigenic potency after storage for 2 to 3 months, has not been substantiated. Eight different preparations, stored at 8° to 10° C. and tested periodically, were highly potent at the end of four and one-half years.

For bibliography see the original article.

A NOTE ON THE CLAIM THAT AGGLUTININS ARE LIPOIDAL IN NATURE

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Charles Krumwiede and W. Carey Noble

The claim that agglutinins could be extracted from sera by the use of lipoidal solvents, was called to our attention by the address of Jobling on the relation of lipoids to immunity. This claim was made by Stuber, who believed he had demonstrated that he could extract the agglutinins from an immune serum with petroleum ether and that the addition of the extracted substances to normal serum would result in an agglutinating value practically equal to that of the unextracted immune serum. He claimed, also, that the serum of normal animals became strongly agglutinative after the animals had received intravenous injections of such extracted substances.

We have attempted to duplicate the results of Stuber without success. Experiments were carried out with serum as separated from the clot and with dried serum. Extraction was done with ethylic ether in some experiments and with petroleum ether in others.

Conclusions

There is nothing in the above experiments to indicate that agglutinins are lipoidal in character to the extent that they are soluble in lipoid solvents. In no case were we able to reduce the titer of extracted sera beyond a reduction which is explainable by the manipulation of the serum. In no case was there a transfer of agglutinins by the extracting solvent where we could not at the same time demonstrate a transfer of antigenic proteins as demonstrated by the use of the precipitin test with anti-horse serum. The methods employed were relatively simple. This was desirable in view of the destruction of the antibody by more complex methods. However, the methods used would suffice to demonstrate the solubility of agglutinins in the solvents used, if they were soluble in these menstrua.

For bibliography see the original article.

AGGLUTININ ABSORPTION

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CHARLES KRUMWIEDE, GEORGIA COOPER AND DOROTHY J. PROVOST.

I. INTRODUCTION

The absorption of agglutinins was first observed by Gruber and Durham (1896) or at least they noticed that during agglutination, there was diminution in the amount of agglutinins—"gebunden," "zerzetzt" (Gruber). Bordet (1899) employing normal serum which possessed agglutinating properties, showed that the absorption was specific; the addition of the cholera vibrio resulted in the loss of the agglutinins for this bacterium but not those active against the typhoid bacillus and vice versa. Hahn and Trommsdorf (1900) proved that agglutinins were actually absorbed, not inactivated or destroyed. By treating the agglutinated bacteria with weak alkalis or acids, they were able to recover at least some of the agglutinins. Eisenberg and Nolk (1901-1902) investigated the quantitative factors of the phenomenon of agglutinin binding as well as the effect of chemical and physical agents upon the serum or antigen and the influence of such treatment upon absorption.

Gruber and Durham and especially those that followed them, appreciated clearly that serum agglutination was only relatively specific; that a serum would agglutinate not only its homologous bacterium but also related varieties or even unrelated varieties. These heterologous types were usually agglutinated in lower dilutions than was the homologous type. In exceptional instances, however, surprising and apparently paradoxical results were obtained. Thus, the serum from a case of enteric fever might agglutinate equally well two or even more of the bacilli of the typhoid-paratyphoid group. In some instances, a variety that was not the infecting type, would be agglutinated to the greater degree. Some observers suggested that such paradoxical results were due to overlooked secondary infections. This explanation, however, was not acceptable for some of the apparently contradictory results were obtained in cases in which careful and repeated bacteriological examination excluded this possibility.

To eliminate such difficulties, Castellani (1902) suggested the practical application of the experimental knowledge on agglutinin absorption which had been made available by previous observers. To demonstrate the practicability of such an application, he presented the absorption results obtained with serums of animals immunized with one or more bacterial types. He advanced the following criteria as the basis of such practical application:

1. A monovalent serum absorbed by its homologous culture loses its agglutinating capacity not only for the homologous culture but also for such heterologous strains as it may have agglutinated. Absorption by a heterologous strain results in the loss of agglutinins for this strain without the loss of the agglutinins for the homologous strain.
2. A bivalent serum when absorbed by one of its homologous cultures loses the agglutinins for this culture and not for the second and vice versa; when absorbed by both, all the agglutinins are removed.
3. These results can be utilized in the diagnosis of mixed infections; also in the identification of an organism.

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[1] We are indebted for assistance to Allen Johnson, Katherine Cox, Lucy Clark, Elizabeth Waterman and Hortense Lebenstein.

Although agglutinin-absorption has been the subject of numerous papers, it is surprising to find after the lapse of over twenty years how many misconceptions still persist; first, as to the validity of Castellani's conclusions; second, as to the practical application of what is valid; third, the method of application; fourth, the limitations of the method, and fifth, the deductions that may be drawn from the results of its application.

The difficulty lies primarily in the fact that the literature on the subject is large and frequently contradictory.

No attempt at an exhaustive survey of the literature has been made, hence no priority in observation is necessarily implied where no references are given.

In presenting the subject the application of the absorption method to the identification of bacterial types is considered first. The validity of Castellani's deductions as regards diagnostic application is left for a later section.

As a basis for further discussion we should restate Castellani's theorems, thus: the culture used for injection absorbs from the resulting immune serum all the agglutinins, the production of which was due to the specific antigenic stimulus of this bacterium. The corollary of this is that the agglutinins in a serum, not absorbable by the homologous culture are not due to the specfic stimulus of the antigenic matrix of this bacterium. Under appropriate conditions a culture which absorbs all the agglutinins stimulated by and absorbable with the serum culture, is similar to the serum culture. A culture which absorbs only a part or none of the agglutinins is different from the serum culture.

These theorems, as restated, are discussed in detail in the following sections. The division of the subject matter under the headings given, is artificial and some overlapping and repetition results. In spite of these drawbacks, some such subdivision is necessary if emphasis is to be placed upon the essential sources of error and misinterpretation.

In the consideration of the subject of agglutinin-absorption it must be kept in mind that agglutination is a two-phase phenomenon; first, combination of agglutinin and bacterium; second, flocculation through physical action.

Many important points are not directly available under the particular heading of agglutinin-absorption but are scattered in articles on the study of different bacterial groups and types. None of the books on immunity or serology more generally used nor any of the sections on agglutination in the larger systems serves as an adequate source of information. In most of the texts, agglutinin-absorption is described primarily in relation to the theoretical aspects of agglutination. Other writers make statements which though true in themselves are wholly misleading when read with their context.

The absence of any adequate review has suggested an attempt to summarize what we consider the essential facts and fallacies of the agglutinin-absorption method. We felt that this attempt was justified by the experience gained in an extensive application of the method during the last five years. This experience has covered many groups of bacteria such as the typhoid-paratyphoid group, meningococci, streptococci, pneumococci, B. influenzae, and B. pertussis; also a discussion of these results with other workers and the privilege of verifying the nature of unusual types of bacteria which have been submitted to us. A study of the more recent literature has been a further stimulus in this direction.

In undertaking such a summary we are basing the conclusions, as far as possible, on our own observations. Some of the examples utilized in the discussion have accumulated during our work on different bacterial groups. More recently, intensive work has been carried out with the one aim of eliciting further examples. The selected number of protocols which are presented later give little indication of the entire amount of work. Many of the examples given appear to us much more striking than similar ones which have been presented in the past. We appreciate that many of the points discussed have been raised by others either on the basis of actual observation or speculation.

II. THE SIGNIFICANCE OF THE ABSORPTION OF COMMON OR GROUP AGGLUTININS

Summary: Direct agglutination may or may not be a reliable index as to the nature of a bacterium as this agglutination may be due to common agglutinins. The absorption of common agglutinins may be employed for tentative separation into groups, but such separation has no inherent validity because such grouping may vary with the number and quality of the serums employed. Individual cultures may be erroneously classified by such a method. The absorption of common agglutinins is not a criterion of likeness. The absorption of specific agglutinins must be used to determine agglutinative identity. Information concerning the specific absorptive capacity is a necessary criterion for conclusions as to the constancy or significance of fundamental agglutinative characteristics.

III. THE ABSORPTION OF "POLYVALENT" SERUMS

Summary: The absorption of a polyvalent serum may give tentative information of some value in the case of related cultures if the cultures used for immunization are also available for the test. To obtain information that will be of any considerable value, however, it becomes necessary to test all the cultures, including the cultures used for immunization, after each absorption. The value of the results will depend on how much the animal injected has responded with the production of specific agglutinins for each of the cultures injected. This is difficult to determine with any degree of certainty. These difficulties become proportionately greater, the larger the number of cultures used for immunization.

The use of a serum for absorption when one does not possess the cultures used in the production of this antiserum is open to the objection that one is working with unknown reagents.

In either case cited above the sources of error are essentially the same as those discussed under the absorption of common agglutinins.

If the method is applied with all possible safeguards the work becomes as great or greater than that involved in the preparation and use of monovalent serums to determine specific absorption capacities.

IV. THE ABSORBING DOSE, THE TIME OF CONTACT, ETC.

Summary: It is recommended that the minimal absorbing dose of the homologous bacterium be determined for each serum. One and one-half to three times this dose is generally a satisfactory amount to select for carrying out a series of absorptions to determine the likeness or unlikeness of other cultures. In the case of very close relationships or where a culture shows variations from the normal, comparison of the results with different sized doses or the use of a less than complete absorption dose, may be necessary.

V. TESTING THE DEGREE OF ABSORPTION

Summary: As a general rule, it is highly desirable to test an antiserum after absorption as low as 5 per cent. of the original serum titer (highest dilution showing good agglutination). In some instances it may be desirable to go below this level. To obtain an adequate picture of the results of absorption the absorbed serum should be tested in dilutions covering the whole active range of the unabsorbed serum.

Tests throughout the whole serum range are especially necessary where titration of the absorbing doses is used as an accessory means of interpretation. Another reason for testing the whole serum range is the occurrence of zone-phenomena (next section). Lesser degrees of absorption to 25 to 50 per cent. of the titer are proportionately less adequate. The absorption to 25 per cent. may suffice for broad grouping but it may fail to separate very closely related varieties. Conflicting results using this criterion may be due to variations in dosage which would not be noted in the absence of tests in the lower dilutions. The adequacy of any selected percentage will be more stringent if one selects as a criterion of serum titer the highest dilution showing complete agglutination. There are many difficulties in applying such a criterion. The use of only one dilution of the absorbed serum as a measure of absorption opens many dangers of misinterpretation and is wholly inadequate.

VI. THE PREZONE PHENOMENON

Summary: The absorption of a serum by bacteria, homologous or heterologous, may exaggerate a pre-existing prezone or may introduce one. This is due apparently in large part to the products of autolysis and to some extent to media constituents. The use of sedimented bacteria resuspended in fresh saline is therefore preferable for the absorbing dose. The prezone, if exaggerated, may lead to difficulties or to actual error when it is overlooked. The occurrence of prezone phenomena is another reason for testing absorbed serum through a wide range of dilutions.

VII. THE RECIPROCAL-AGGLUTININ ABSORPTION TEST

Summary: The fact that culture A absorbs the agglutinins from serum B is no criterion that culture B will likewise absorb the agglutinins from serum A. The method of testing the mutual absorptive capacities of two or more cultures using antiserums for each, is termed the reciprocal absorption test. The reciprocal test is the ultimate method available for determining agglutinogenic likeness or unlikeness. Agglutinogenic likeness as determined by reciprocal absorption is usually paralleled by close similarity in other characteristics. Exceptions, however, do occur, that is, cultures differing essentially in cultural and pathogenic characteristics may be nearly, if not completely, indistinguishable by the reciprocal test.

VIII. THE "RECEPTOR ANALYSIS" METHOD, THE CHARACTER OF FLOCCULATION, ETC.

Summary: We have no definite knowledge as to what underlies the specificity of serological reactions. The concept therefore that each and every agglutinative response or reaction is a criterion of the existence of separate components in a bacterium and similarly distinct agglutinins is purely hypothetical and a "receptor analysis" method based on such conceptions has probably no validity. The method is, however, of value as a means of simple presentation of empirical data to emphasize apparent relationships and to obtain suggestions as to further observations. In this regard, its value is greatest when based on reciprocal reactions obtained with antiserums and their homologous cultures as essential reagents. Additional observations on the character of clumping with the use of heated and unheated antigens and on group reactions may serve to yield additional suggestive data. Such additional modes of observation cannot be used as substitutes for the basic reagents antiserum and homologous culture. The "double receptor-analysis" method has no advantage over the regular absorption method when applied simply as a means of identification or differentiation. The method may, however, yield data which is of value in attempting to explain difficulties in differentiation of certain allied bacteria.

IX. Preliminary Summary: Likeness and Unlikeness

Thus far we have considered the application of agglutination and agglutinin absorption as applied to so-called pure cultures. In this mode of application we think of a culture as consisting of only one variety of bacterium having certain specific agglutinogenic agglutinative and absorptive capacities which permit its identification or separation from cultures of other varieties. Not uncommonly therefore we speak of it as a bacterial type. In the usual sense, this implies that we think of a pure culture as an entity. Naturally this is not actually the case as a culture consists of many individuals of many generations. If we do speak of such a culture as a bacterial type we may be assuming either that each of the individuals has essentially the same specific serological qualities or that the type characteristics depend on the characteristics of the mass and not on the individuals.

Agglutination and agglutinin absorption as generally used for indentification or classification purposes are applied to cultures as though they were entities. It seems desirable therefore to summarize the facts thus far presented in terms of bacterial cultures before considering the question of the constituents of the culture, that is before discussing whether a culture is homogeneous or not from the serological standpoint.

First there are the reagents themselves. There would seem to be no need of discussing the reliability of the cultures used for study, their source, purity, etc. In the discussion thus far we have not considered variables; such as differences in agglutinability, absorptive and antigenic capacity, etc. among cultures of like varieties. These factors will be considered in succeeding sections. The summary that follows assumes, therefore, for purposes of discussion that the culture is a constant.

The antiserums to be utilized require some comment. One may be compelled in ordinary routine work on the commoner bacterial varieties to use "stock" antiserums obtained from other laboratories. Too frequently such antiserums are utilized in investigative work without regard to the species of animal from which they are obtained, without a knowledge of the "normal" antibody content and without the cultures employed for immunization to serve as a control. From the facts thus far emphasized this method is evidently far from desirable and in the ultimate analysis means that the work done with such antiserums is really with unknown reagents. In fact, examples have been given which show that the contingency is not remote that an investigator using antiserums in good faith may be unwittingly working with group agglutinins. The necessity of a knowledge of the species of animals employed may be emphasized by reference for instance to the broad agglutinin content which has been observed by Park and others in antidysentery serum obtained from horses. The preparation of antiserums by the worker with due regard to the species employed and with all necessary controls is therefore emphasized again.

On the whole the rabbit is the animal of choice for the production of agglutinating antiserums. Some have recommended other species, sheep for instance, as yielding a lower content of group agglutinins. Our own experience does not indicate that either sheep or goats yield better serums in this regard. As individual animals vary to a considerable extent it would require more extensive comparative work to reach a definite conclusion. On the whole moderately young animals seem more desirable than old animals. Storing of antiserums may be advantageous as the normal and common agglutinin content seems in some instances to fall rather quickly.

Even where one prepares one's own antiserum oddities may be encountered as we have noted. It may be necessary, therefore, to prepare another antiserum for control purposes wherever any peculiar results are observed. Should an antiserum show marked cross-agglutination, the degree of which seemingly is out of proportion to the probable degree of relationship of the heterologous bacteria agglutinated, it may be desirable to prepare other antiserums. Another antiserum may show very much less common action and thus eliminate the necessity of absorption tests which would be required with a more broadly acting antiserum. A point often lost sight of is the possibility of intercurrent spontaneous infection of the animals employed by a bacterium related to the one used for injection. We have encountered this several times. In one instance this resulted from accidental cross-infection from animals injected with paratyphoid cultures for virulence tests to animals used for serum production. That is the infection was caused by a bacterium virulent for rabbits but not a bacterium ordinarily occurring in them. That this contingency is overlooked is frequently seen in the literature where workers draw certain theoretical conclusions from results which may well have been due to an intercurrent infection of the rabbit used for injection.

That the titer of the antiserum is an important consideration should need little emphasis after the previous discussion. It should be as reasonably high as is consistent with a duration of immunization which will not unduly raise the group agglutinin content. The working range of an antityphoid serum of a titer of 1 : 800 is not as satisfactory as one of 1 : 8000. On the other hand unduly potent antiserums are not desirable as the absorbing doses must be inexpediently large.

The pooling of two or more antiserums for the same bacterium or for presumably similar bacteria has been commmented on and is to be condemned.

The use of the group-absorption method is only justifiable as a preliminary means of differentiation. No study of two bacteria by the group absorption method however extended will suffice to demonstrate their likeness.

The use of the polyvalent antiserums for investigative work is not to be recommended even where the homologous serum cultures are available. The use of polyvalent antiserums where the homologous serum cultures are not available has no justification. Their use resolves itself into a group absorption method with unknown reagents and its value is only that of the group absorption method.

If the group absorption method is employed for so-called "receptor analysis," it should be as a secondary and not a primary means. An apparent but not real exception will be noted later. The degree of absorption of the agglutinins and the standardization of the absorbing doses require attention. The minimal absorbing dose should be determined by actual trial. In general two to three times the minimum required when the homologous serum culture is used is a satisfactory absorbing dose to employ with other cultures, so that unimportant variables shall not confuse the results. The testing for the reduction of the agglutinin content to 5 per cent. of the original titer generally furnishes a safe criterion of agglutinogenic similarities or differences. Under certain circumstances titration of the absorbing doses and tests in dilutions lower than 5 per cent. of the original titer may be desirable.

Due attention must be paid to the introduction of phenomena caused by substances which accompany the absorbing dose and act as a "third colloid." The development of marked prezone reactions, the inhibition of spontaneous agglutination etc. have been discussed. Similarly disturbing reactions may be due to peculiarities in the serums.

Complete information as to likeness or unlikeness of two cultures is only obtainable by reciprocal tests with the cultures and their homologous antiserums. This means that a complete classification of a set of cultures is only possible where one prepares and tests antiserums for each of the cultures in the series. This is not always expedient and in many instances a reasonable certainty may be obtained with a less complete series of tests. This is not open to criticism unless it be presented or interpreted as complete and final evidence.

The reciprocal method of testing gives a reliable mode of determining the dominance and subsidiary position of the members of a group of bacteria that have very close relationship. Any attempt at an analysis of the presumable receptor or antigenic content or mosaic of a bacterium should be based primarily on such a method utilizing cultures and their homologous antiserums as the test reagents.

The necessity for reciprocal tests to determine complete identity indicates that in the last analysis the antigenic or antibody stimulating function of a bacterium is the essential one, that agglutination and agglutinin absorption are in fact only measures of this function. This conception implies that a bacterium is capable of absorbing all the agglutinins stimulated by it, and that the actual serum culture is the only control required for measurement. This conception must be considered fundamentally valid and absorption work primarily should be based on such a criterion even though exceptions may occur as will be noted later.

Usage has led to the application of the terms antigen, antigenic, etc., to the reacting reagent. Thus the bacterial suspension is referred to as the antigen, or a more extreme example is the use of the term "antigen" to designate the non-specific reagent in the Wassermann reaction. This has led to some confusion. Thus an observer on the basis of agglutination and agglutinin absorption using a few antiserums may conclude that he has demonstrated the existence of antigenic groups among cultures of a definite bacterial variety, as B. typhosus or B. paratyphosus B. Actually this is not so. As has been emphasized, an actual antigenic grouping would have to be based on the primary criterion, the antibody stimulating qualities of a bacterium.

A study of the character of the clumping of bacteria may yield data of theoretical and practical interest. Such observations do not add validity to the group absorption method. The character of agglutination may give presumptive evidence but does not serve as a complete substitute for the more certain agglutinin absorption results.

The fundamental requisite in agglutination and agglutinin absorption is therefore a culture and an homologous antiserum about which one possesses as full a knowledge as is obtainable.

X. VARIATIONS—CRITERIA

Summary: The ultimate criteria in the investigation of the occurrence of serological variations and of the kind or direction of such variation should be the study of the antigenic properties of the culture and its variants. This means the preparation of a homologous antiserum for each culture and for each strain or variant under study and a measurement of the antigenic response in each case by reciprocal agglutination and agglutinin absorption tests.

XI. VARIATION—PHYSICAL PECULIARITIES AND COLONY VARIANTS

Summary: Antigens from some cultures may show an instability which is reflected in their greater agglutinability or in actual spontaneous flocculation. Antigens from certain colony variants usually show this. If overlooked this may introduce difficulties in agglutinin-absorption or lead to errors in interpretation. Different colony types, rough, smooth, mucoid, etc., may be obtained from many bacterial types, the antigens of which differ in their characteristics. Such variants may give different results when their absorptive capacities are investigated. Where marked differences occur between the rough and smooth strains from a culture this is seemingly not a necessary concomitant of colony variation as similar differences can be elicited between two colonies of the same type. The rough strain, however, tends to show a generally lower absorptive capacity than does the smooth strain. The few mucoid types studied showed marked peculiarities especially in regard to lower agglutinability and low absorptive capacity. Only one "mucoid" antiserum was studied. The results indicated that the mucoid type differed antigenically from the rough and smooth types of the same strain. Whether this difference is an inherent characteristic of the mucoid type cannot be determined from one experiment. The failure to obtain differences with other types which uniformly paralleled the colony characteristics, indicates that the antigenic differences observed may be due to other factors than the mucoid characteristic alone. Some who have studied this phase have obtained more uniform agglutinative and absorptive differences between the colony types. It seems that when such marked differences occur other variation factors are associated. These variation factors which are indicated by antigenic changes, however, are not necessarily associated with variations in colony morphology.

XII. VARIATION—SEROLOGICAL HOMOGENEITY

Summary: Bacterial cultures may show a dual composition. The two components have been designated tentatively as "specific" and "group." This terminology is descriptive of the two components when they are studied in the group of closely related paratyphoid varieties. The essential agglutinative and absorptive differences between these related bacterial types depend on the specific component or fraction. The cross-agglutination and cross-absorption depend on the group component or fraction. This fact indicates a practical application namely, the use of the specific component or fraction in bacterial identification or classification or as an antigen in diagnosis agglutination work.

Certain limitations of this method as an ultimate substitute for absorption, are discussed. Even if pure component antiserums are not prepared a knowledge of the relative constitution of the cultures and antiserums is of great help in preventing false interpretations. The two components of any culture differ sharply from each other in regard to their reciprocal agglutinative and absorptive characteristics. Each component or fraction, however, possesses some of the characters of the other component or fraction; the group variety possesses those of the specific variety to the greater degree. This may be due to the pecularities noted in the breeding of these two components, the group variety showing a marked tendency to split off specific components, whereas the specific variety tends to breed pure. The dual constitution cannot be made to explain all group reactions. This duality of composition has been observed with the organisms of the paratyphoid group. Both components have been isolated from original material and from cultures. In some instances only one component has been found. Cultures yielding rough colonies have a duality similar to the smooth. Thus far a completely like condition has not been demonstrated for B. typhosus. The phenomenon of duality probably occurs in other bacterial groups, and should be considered and investigated in any classificatory study.

XIII. Variation—A Phenomenon of Degradation

Summary: Either stock cultures, strains from cultures or directly from infected persons may show evidences of loss of certain characters, which are characteristic of the bacterial race from which they originate. Such strains, therefore, have been termed "degraded variants." The degradation may vary in degree. The extreme degree among members of the typhoid-paratyphoid group is characterized by lessened or lost motility, a loss in agglutinability and an agglutination which occurs in fine clumps; also a marked loss in antigenic capacity. An antiserum homologous to such a degraded variant, may agglutinate the original culture in a higher dilution than the variants and absorption of such a serum by the degraded variant may fail to remove all the agglutinins active against the original culture. This is an exception to the general conclusion that a culture or strain will absorb all the agglutinins due to its specific stimulation. With antiserums for degraded variants of the typhoid-paratyphoid group, degradation may or may not be associated with a loss of specificity in that heterologous types are able to absorb the agglutinins from the degraded variant antiserum active against the degraded variant or even those active against the original culture. In some instances the degradation has resulted in an apparent increase in specificity. With B. proteus varieties it has been reported that an increase in specificity results from what is apparently a degradation process. Degradation is usually also followed by a loss in virulence. Colony variation may or may not be an associated phenomenon.[1] Although the phenomenon of degradation has been studied mainly with motile bacteria where changes in motility are associated with the change even if not a factor in it, similar degradation apparently occurs with non-motile species. The details of the changes which accompany degenerative changes among non-motile varieties require further study.

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[1] Since the above summary was written, reports by Arkwright (Jour. Path. and Bact., 1924, 24, 36) and Burnet (Jour. Exper. Path., 1924, 5, 251) have appeared. They use R (rough) and S (smooth) as synonymous with O and H the designations used by Weil and Felix for small and large clumping. We believe that they do not appreciate all the factors involved. Our results show that such a parallelism may occur, but it is not a necessary one.

The loss of specificity frequently shown by the degraded variant of the typhoid-paratyphoid group indicates that those characters which are associated with fine clumping underlie to some extent the phenomenon of group agglutination. Whether this factor in group agglutination is distinct from or related in some way to the group reactions due to group components cannot be definitely stated. The fine clumping factor is seemingly the specific element with some paratyphoid types. There is insufficient data to warrant a conclusion that in such instances the degraded strain originates from the specific component.

The failure of reciprocal absorption between a type and its degraded variant introduces a difficulty in interpretation, that is, whether one is dealing with actually different race types or with such a type and its degraded variant. When the culture shows other characters of degeneration the probability is in favor of the latter presumption. This is a distinct practical difficulty which may be encountered in routine examinations. If the degraded variant reverts to its race type, this will eliminate the difficulty.

Some degraded variants have shown only an apparent loss of certain race characters; these we have characterized as "vertical variants." Others have shown the apparent acquisition of characters not possessed by the race type; these we have termed "lateral variants."

XIV. Variation—In the Host or in Transfer from Host to Host

Summary: Certain definite types of serological variation may be accepted as occurring in the infected host. Most of the reports on variation in the host and in the transfer from host to host are only hypothetical and other hypotheses might be equally applicable. More carefully detailed serological observations, which will satisfy all the necessary criteria are needed, before any conclusions are justified as to the frequency, direction and degree of serological variation which bacteria undergo in the host or in the transfer from host to host and the limitations this places on conclusions drawn from the practical application of agglutination and agglutinin absorption.

XV. Variation—The Limitations It Imposes

In this summary we shall consider only those variations which, so far as we know from the evidence available, may occur under "normal" conditions, that is, variations which have been observed with strains obtained directly from the host or from cultures maintained by those methods, which presumably are adapted to an approximately natural mode of multiplication. It is the variations which occur under these conditions which are likely to lead to embarrassment in the practical application of the absorption method. As has been noted, very extreme variants apparently may be obtained by prolonged cultivation in broth without transfer and by other equally artificial means. Such variations have biological and some practical interest in the study of variations and its direction. Naturally such extreme variations may occur under normal conditions and this must, therefore, be kept in mind. At present, however, observations indicate that such extreme variation is comparatively infrequently encountered and consequently is not often a factor in the practical application of the absorption method.

We have emphasized again and again the postulate that a completely positive reciprocal agglutinin absorption test is the ultimate criterion of serological likeness or identity of two bacterial cultures or strains and that the absence of a completely positive reciprocal reaction is a definite criterion of unlikeness. This postulate is fundamentally valid, but its unreserved application would not serve the actual practical purposes for which the agglutinin absorption test is usually applied. Thus the data on variation show that if the postulate were unreservedly applied, we should be confronted with the necessity of assigning to different groups the variants, which are obtainable from a B. paratyphosus B. culture for instance. Such variants are actually serologically different and the observed differences may correlate with other differences; nevertheless the name B. paratyphosus B. applies to all, as they fall within the observed limits of this variety. At present there is no immediate purpose to be served by adding a varietal name to designate them.

If such definite types of B. paratyphosus are really races, it is obviously necessary to determine the potentialities for variation of other bacterial groups, if we are not to be led astray. Such information is relatively limited, and its acquistion may be of considerable aid in interpretation. Thus reciprocal differences may be noted and a number of cultures may fall, therefore, into dominant and subsidiary groups. The problem is, whether these findings are a result of inherent and constant race type differences or whether the subsidiary appearances are simply manifestations of the range of variability of a single race.

Apart from the question of serological classification as such, there is the danger of misinterpretation in more immediate practical work. Assume that in investigating an epidemic of paratyphoid fever, we isolate (as an extreme possibility) different variants from different individuals. Having a knowledge of the limits of type variability we should not be confused or fall into the error of ascribing the epidemic to two or more types. The isolation of variants having degraded character introduces a distinct difficulty in identification. The correlation of all the characters will usually give a reasonable certainty. Complete certainty can be obtained in some instances only if reversion to type fortunately occurs.[1]

In this connection the general application of the agglutinin absorption test as a criterion in epidemiological studies, requires further comment. Thus we showed during the last pandemic of influenza that the cultures of B. influenzae obtained from cases were in general different each from the other. We concluded that such heterogeneity could not exist if B. influenzae were the primary etiological agent of the pandemic. That is, the causative agent being transferred rapidly from case to case, if inferences may be drawn from previous experience, could not be conceived as varying to this extent. Do the observations on variation, here described, suggest that such a conclusion was not warranted? Assume that B. influenzae were a defined race and that its range of variability were far wider than any thus far observed. Is it possible that, even under these conditions, we should have observed practically no likeness, or at least no similarities which would have suggested at least the presence of variations such as we have described? Even the most radical proponents of variation in the host, such as Seligman, predicate serological stability at the height of an epidemic. As a matter of fact, there is little likelihood that variants from the type race norm would be in the majority. Those that were encountered could be recognized in many, if not most instances, as variants from the epidemic race type if appropriate means were employed. Although somewhat outside the present subject, it might be well to emphasize that the reverse of the above epidemiological argument is not necessarily conclusive. One and the same bacterial variety might be isolated from a series of cases and this variety might still not be the primary etiological agent.

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[1] We have just encountered a convalescent case of typhoid fever whose feces contain no typical B. typhosus but only what correlates in every way with the degraded variants described in the previous section.

A knowledge of the potentialities of bacterial variation is also evidently of importance in carrying out agglutination tests with patient's serum for diagnostic purposes. The possibilities of more clean cut differential results with the use of the specific component has been referred to. The possibilities of error in the employment of group or degraded variants are evident from preceding discussions.

It is obvious that the value of antiserums for routine identification purposes may be influenced likewise by the character of the cultures employed for injection and that the use of variant cultures may result in errors. These factors have a direct practical bearing on the production of vaccines and antiserums.* More detailed study of these factors in regard to the production of protective substances would be required, however, to determine all the limitations due to these factors.

In general criteria outlined in the preliminary summary (section IX) are invalidated in no way by the facts presented on variation. Variation only imposes certain limitations which must be observed, and agglutinin absorption results must be interpreted accordingly.

There is an apparent limitation in the opposite direction, in that at times the agglutinin absorption method is insufficiently delicate. Examples of this have been given; the failure to separate a presumed B. abortus culture from a presumed B. melitensis culture, likewise the close absorption relationships of B. typhosus, B. pullorum and B. sanguinarium. As has been noted this limitation has some relationship to the phenomena which operate in the loss of specificity through degradation.

XVI. May a Culture Be Unable to Absorb All the Agglutinins From Its Homologous Antiserum

Summary: We have restated one of Castellani's theorems thus: the agglutinins, the production of which was due to the specific stimulus of this bacterium.

This restatement is necessary as an antiserum may contain "normal" agglutinins without regard to the specific stimulus. Some apparent exceptions to this theorem are noted. Actual exceptions have been observed thus far only in the case of degenerate variants, which may be unable to absorb all the agglutinins whose production it stimulated.

XVII. Technic

See original article.

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* Difficulties in obtaining an adequate antibody response in horses for the production of curative antiserum are encountered from time to time. We have recently encountered considerable trouble in preparing typhoid vaccine of sufficient agglutinogenic value to pass the standard requirements. It might be worthy of note that the serum of the rabbits injected with these defective vaccines also showed an almost complete lack of complement fixing antibodies. Such difficulties are referable to degradation in the stock cultures.

XVIII. AGGLUTININ-ABSORPTION APPLIED TO THE SERUM OF INFECTED PERSONS; DIFFERENTIAL DIAGNOSIS OF DISEASE

Summary: There is no other means of measuring the specific agglutinin content of a serum except by using the bacterial type which is known to have stimulated the production of the agglutinins in a serum even though this means may not always be adequate. There is no basic validity therefore in a procedure which assumes the reliability of determining the infecting bacterium or bacteria by testing the absorptive capacity of several cultures on the agglutinins present in the patient's serum for each of such cultures. Such a procedure does not take into account all the possible alternatives. The observation of the character of the clumping and the use of the specific component for test purposes may give strongly suggestive results. The probable alternatives in such methods are discussed.

Bibliography: See the original article.

A STUDY OF THE SPECIFICITY OF THE ABSORPTION OF ANTI-BACTERIAL PRECIPITINS

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Charles Krumwiede and Georgia M. Cooper

In planning some experimental work on the immunological identity or non-identity of certain bacteria, it was found that a suitable agglutination antigen, that is, a stable suspension of uniform turbidity, could not be prepared from the cultures under consideration. This difficulty as is well known occurs with some bacterial types. The question therefore arose whether under the circumstances we could employ the precipitin reaction. Should sharp differences be noted with this reaction, non-identity would be established. Should, however, the antigens of two or more cultures be found to give equal or nearly equal precipitation with an immune serum it would be necessary to resort to the antibody absorption method to determine the ultimate identity or non-identity of such strains. How far would such absorptions reveal specific differences?

This question was raised because of the well known tendency of the precipitin-precipitinogen complex to bind immune bodies of all types as well as complement; a phenomenon utilized by Gay, Chickering and by Weinstein to separate the antibodies of immune sera. The question of the specificity of such an absorption has apparently received little attention. Chickering found that an antigen of pneumococcus, type I or type II, removed only the immune substances for itself when a bivalent pneumococcus serum was employed. As these types are relatively very distinct and as they "cross-precipitate" to only a slight degree, these observations are of no significance as regards the question we have raised. Although we have not reviewed exhaustively the existing literature on bacterial precipitins, we do not believe that the fundamental question of the specificity of precipitin absorption has been studied with regard to bacteria which are closely related and therefore give similar or nearly identical reactions with precipitin sera.

We were in possession, fortunately, of cultures and serums which were especially suitable for a study of the above question. Smith and Ten Broeck called attention to the extreme degree of cross agglutination between *B. typhosus* and the avian types *B. sanguinarium* and *B. pullorum* when an immune serum for any one of these varieties was employed.

Extending these observations to the precipitin reaction we found that an anti-typhoid serum would precipitate the antigens of the above three types equally in the same dilutions and for the same periods. The similarity of precipitation was so close that the strains could not be differentiated one from the other by this reaction. We have also found that with an anti-pullorum serum similar cross reactions occur. Evidently, therefore, the serums and antigens of the three bacterial types mentioned above offer suitable reagents in testing the specificity of precipitin absorption. As a control, an antigen of *B. paratyphosus A* was used because antigens from this type were precipitated by the anti-typhoid serum in relatively low dilutions only. This type was therefore easily differentiated from *B. typhosus* by this reaction.

It was planned to carry out the absorptions in two ways; first, by utilizing the precipitin antigen; second, by utilizing the bacterial suspensions as is done in agglutinin absorptions.

The precipitin antigens[1] were prepared according to the method we have previously reported.

The technique is given in the original article.

The size of the absorbing dose in relation to the specificity or non-specificity of the results was given careful consideration.

Our inability to standardize adequately precipitin antigens may be a factor in the non-specificity shown with absorption by antigens which precipitate equally.

The absorption of precipitins from the immune serums by the use of precipitin antigens, or even by whole bacteria, was specific only within narrow limits. With a heterologous type which gave little precipitation, the absorption by the antigen of this type removed only the precipitins against itself. In the case of the heterologous types giving profuse precipitates, absorption by one of these types resulted in the complete removal of all the precipitins, even those active against the type homologous to the serum.

Assume in the case of the above cultures, that satisfactory agglutinating antigens were impossible of preparation, and reliance had to be placed upon the precipitin reaction. If we allow this assumption, it is obvious that the precipitin absorption would have given unsatisfactory results where its application would be required because of failure of differentiation due to cross-precipitation and would have given satisfactory results where it was not required because differentiation was evident on direct precipitation.

The results thus far recorded as regards non-specificity of absorption by the precipitin antigen itself were more or less expected. The result with absorption of precipitins by the bacterial bodies was so unexpected that it raised the question as to how much these sera cross-agglutinated and consequently what influence an equal absorbing mass of bacteria would have on the agglutinin content.

When the effect of a similar mass of bacterial on the agglutinin content of the serums was investigated, it was found that absorption of the serums by the homologous bacteria, using the same masses as in connection with the precipitin tests, reduced the specific titer only to a slight degree. Absorption by a heterologous strain, in the doses noted, also had relatively little influence on the height of the specific or group agglutinations. However, if the group titer was low, the heterologous strain might serve to remove the agglutinins for the absorbing type, but would leave the specific titer nearly unchanged.

The non-specific results obtained with precipitin absorption in the above experiments induced us to try other related varieties of bacteria and their antisera in the attempt to verify the results recorded in the original article.

The agglutinin-absorption technic leaves us a practical and relatively wide working range as regards dosage. The precipitin absorption method on the contrary, as the experiments indicate, has a narrow range and may fail to reveal distinct differences, especially where strains are closely related, even with scrupulous regard as to the size of the absorption dose.

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[1] The term antigen is used in the sense of a reagent. The ability of antibody stimulation is not implied.

CONCLUSIONS

A precipitin absorption method has only a limited application in the differentiation of closely related types of bacteria which, because of such relationship, show marked cross-precipitation. The tendency in such a case is toward non-specific results; that is, the bacterium, heterologous to the serum, in removing the precipitins active against itself may also remove the precipitins active against the homologous type. Where the cross-precipitation is less marked, more specific results tend to appear after precipitin absorption.

The results are the same whether a precipitin antigen or the bacteria themselves are used for absorption.

The results may be influenced by our lack of a suitable method for the standardization of precipitin antigens of bacterial origin. The conclusions as given, therefore, are stated in terms of this limitation.

The non-specific results obtained may be referable, in some degree, to the narrow working range of precipitating sera.

The inadequacy of the precipitin reaction as a primary or single method for the differentiation of related bacteria, which are serologically still unclassified, is again emphasized. While similar failures in differentiation may occur with the agglutination reaction, such failures may be easily corrected by resorting to the absorption method.

For bibliography see the original article.

PRACTICAL EXPERIENCE WITH THE RAMON FLOCCULATION TEST IN DIPHTHERIA.

Olga R. Povitzky.

This test was given a full trial in the early spring of 1924, and the results were encouraging[1]. Since September 1924 the Ramon test has been employed regularly for following up the progress of our horses immunized against diphtheria, and closely checked by guinea pig titrations. At first we used a toxin which contained 4 L + per one cc. of toxin. With this toxin the tests were made with from 2.5 to 1 cc. of toxin per tube depending on the strength of the sera tested. Later on, with a toxin of 11 L + per cc., we used exclusively 1 cc. per tube. The varying amounts of the tested serum were deposited first in the regular agglutination test-tubes and followed by 1 cc. per tube of the toxin (previously carefully titrated by guinea pig and Ramon tests). The contents of the tubes were well agitated and put in the water bath at 50° C. With the strong toxin the tests had to be watched closely as the reaction in the indicating tube occurred very promptly (from ten minutes to one hour). With the weaker toxin, however, the reactions were not as prompt and took from one to six hours and sometimes longer. The great essential for this test is therefore a strong, stable toxin titrated for its L + value. The stronger the toxin is, the prompter will the result be. The economy in money and time, resulting from the use of this method for estimating the values of antitoxin and L + toxins, is self-evident. There are, however, slightly over 15% discrepancies (up to 75 units) between the guinea pig and Ramon flocculation tests (as indicated by the table). This leaves out the one very irregular horse. If we include him the percentage is over 22%. Thus on 169 consecutive bleedings of 18 horses there were 36 discrepancies ranging between 50-100 units. In one horse it went up as high as 200 units. The other 6 horses showed discrepancies ranging not higher than 10-25 units (which can be disregarded). Discrepancies ranging above 75 units are met with in a little over 4% of bleedings (outside of horse 87). If we include him the discrepancies above 75 units would be in 7.6% of bleedings. Whether the high discrepancies which usually occur in the highly immune horses (above 400 units) is due to a higher protein content, which influences either of these two tests, is to be elucidated.

Out of 169 bleedings of a total of 18 horses, the Ramon flocculation test, in 7 instances, showed an excess of 75 units over the guinea-pig test.—in other words, 33% more units than shown by the guinea-pig test, out of the same 169 bleedings, the guinea-pig test, in 6 instances, showed excess of 75 units over the Ramon flocculation test—a discrepancy of 16-33 per cent.

Five of these instances were in the bleedings from the same horse. These results were obtained in the first year of application of the Ramon test. After employing the Ramon flocculation test for three years the Writer is more and more convinced that this test is a valuable, reliable agent for testing the progress of the horses under immunization. At present it replaces almost entirely the use of guinea-pigs.

During these three years our studies have shown that the flocculation and guinea pig tests do not present as many discrepancies as they appeared to in the beginning. Thus it was learned that the serums of horses retain

DISCREPANCIES BETWEEN GUINEA PIG AND RAMON FLOCCULATION TESTS ON 18 HORSES.

Horse No.	No. of Bleedings	Discrepancies				Differences in percentages of units of antitoxin in cases of discrepancies above 75 Units
		No. below 75 units	Units	No. showing Units above 75	% above 75 Units	
28	12	3	50–60	0		
33	13	3	50			
42	12	4	About 75	0		
43	12	4	50–100	2	Less than 17%	Twice 33% more for Flocculation Test.
45	12	3	75–100	2	Less than 17%	Twice 33% more for Flocculation Test.
46	5	Very Slight	10–25	0		
49	7	1	45	0		
62	13	3	50–100	1	Over 7.6%	Once 33% more for Flocculation Test.
66	8		20–30	0		
68	12	1	100	1	Over 8.3%	Once 33% more for Guinea Pig.
72	11	3	40–100	1	Over 9%	Once 33% more for Flocculation Test.
78	12	3	70	0		
80	6		10–25	0		
81	2		About 25	0		
84	8	2	50	0		
85	5		10–25	0		
87	12	8	50–200	6	50%	Once 33% more for Flocculation Test.
88	7		10–25	0		Once 33% more for Guinea Pig Test. Once 32.6% more for Guinea Pig Test. Twice 16% more for Guinea Pig Test. Once 23.8% more for Guinea Pig Test.
Total 18	169	38	22%	13	7.6%	all discrepancies up to 75 units.

Total percentage of discrepancies without irregular horse (87) over 15%.
Total percentage of discrepancies above 75 units over 7.5% in 169 Bleedings.
Total percentage of discrepancies above 75 units without irregular horse over 4%.

their characteristics as in the slowness or rapidity of their flocculation reaction. If for instance no flocculation is shown within 1 to 2 hours with a serum which previously had shown reactions within that time, it is probable that the proportion between the toxin and antitoxin is wrong and the test should be made over instead of waiting for a possible later reaction. Some plasmas which do not give reactions in the water bath at 50° C. show a reaction in the incubator (probably some coagulation of the plasma by heat). When the toxin is very strong and the reactions occur very rapidly (from 10-30 minutes) it is best to use a water bath at 37° C.

Toxoids which are made from strong toxins (at least 11 L +) show very good flocculation, while those made from weak toxins show no flocculation. This would show that the Ramon test is not only an indication of the neutral point between an M.L.D. toxin and antitoxin, but also of a saturation point of toxin with antitoxin.

In the Writer's experience it is possible to obtain a Ramon flocculation test with concentrated diphtheria sera, when no acid has been used in the process of concentration. It is not necessary in this case to mix the concentrated with fresh antitoxin serum (Bayne Jones' Method) for the Ramon Test. Our concentrated Diphtheria Sera contain two portions, the one called *B* is precipitated by ammonium sulphate and dialyzed in salt. This portion gives a good Ramon flocculation test. The second portion called *A* is further precipitated out by acetic acid. It is this portion which calls for a mixture with fresh antitoxic serum for the reaction.

Of especial value is the Ramon flocculation test in experimental work. An intensive study of the best means for preparations of strong toxins (which work is in progress now) is made comparatively easy because of this test. Numerous toxins are being prepared and the results determined the same day without the use of guinea-pigs.

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[1] Reference: Povitzky, Olga R., and Banzhaf, Edwin J., Diphtheria toxin-antitoxin titration by Ramon method for practical application. Proc. Soc. Exp. Biol. and Med., 1925, Vol. XXII, pp. 11-13.

SPECIFICITY OF RAMON FLOCCULATION TEST IN SCARLET FEVER

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Olga R. Povitzky

The first paper by the Writer on this subject was presented before the Soc. of Exper. Biol. & Med., May, 1925.[1] The work was continued, and special experiments with various controls established the efficacy of this test for the determination of the potency of scarlet fever toxins and antitoxins. Bleeding of horses injected with scarlet fever toxin were tested each month, and the results, when comparison was possible, usually agreed with those of intracutaneous neutralization tests in children. The curve of the progress of the immunization of the horses with scarlet fever with its gradual rise, fluctuations and drops could thus be studied as efficiently as in the case of horses with diphtheria. Control experiments for the specificity of this test with normal serum, broth, diphtheria toxins and antitoxins, also antibacterial streptococcus and pneumococcus serums were repeated, and the negative results obtained previously were confirmed. The most important control, however, was the 1 per cent. sheep's blood pneumococcus broth used for the growth of the scarlet fever toxin, and incubated at the same time as the toxin. This blood control gave reactions not only with scarlet fever antitoxins, but also with diphtheria antitoxins, antipneumococcus and antibacterial streptococcus serums. That these were pseudo-reactions, due to the sheep blood and fortunately not manifested in the presence of the scarlet fever toxin (due probably to the modification of the blood and change of the *p*H reaction by the growth of the scarlet fever *Streptococcus hemolyticus* in the broth) was proved by the following results: 1. The reactions with the control blood broth appeared in all tubes at the time when there was not even a trace of a reaction with the mixtures containing scarlet fever toxin-antitoxin in the early period of the immunization of the horse. 2. When the reaction between the toxin and antitoxin occurred, it showed only in the neutralization zone, for example, the tubes with a great excess or deficiency of serum remained clear, while the reactions with the control appeared in each tube, irrespective of the amount of serum. 3. The pseudoreactions usually occurred in the form of a film, which could be removed before shaking up.

The following points were found to be essential for the successful result of the test: 1. A toxin standardized for the number of toxic skin doses by the Dick test in children. 2. As a preservative, phenol, preferably the crystals, in 0.3 per cent. solution is used. The ingredients, toxin as well as antitoxin, should be perfectly clear for the test. If they are not clear after centrifugalization they are passed through sterile filter paper. 3. A constant amount of toxin and varying amounts of serum are used for the test. For weak serum the toxin is used in 0.5 c.c. and for stronger serum in amounts of 1 cc. per tube. It is preferable to use large amounts of weak serum, rather than dilute the toxin even slightly. This is true in principle of every

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[1] Ramon Flocculation Test for Determining Potency of Antiscarlatinal Toxin-Antitoxin. Proc. Soc. Exp. Biol. and Med., 1925, Vol. XXII, pp. 426-428.

Ramon test. 4. The optimum temperature for the scarlet fever test is 37° C.; anything above tis point was found to inhibit the reaction. 5. The first reactions to appear in the test tube were found to correspond to those in the intracutaneous neutralization tests in children. 6. The reactions usually appear in from twelve to eighteen hours. With some serums it may be twenty-four hours or even forty-eight hours, especially in case of concentrated serum. Here also, as in diphtheria, the serums of certain horses preserve their characteristics as to the slowness or rapidity of their flocculation reactions. If, for instance, no flocculation is shown within eighteen hours in a case of a serum which previously has shown reactions within that time, it is probable that the proportion between the toxin and antitoxin is wrong, and the test should be made over, instead of waiting for a possible later reaction.

The agglutinin content of four representative scarlet fever horses was studied, because the results may have some bearing on the nature of toxin-antitoxin flocculation. Horses A and C had 85 toxin inoculations, 52 injections of killed cultures and 4 Dochez treatments. Horses 140 and 141 had 26 and 45 inoculations by the Dochez method, respectively. Horse C, which had the lowest antitoxin content by the Ramon test, showed the highest agglutinin titer (1:3,200, higher not tried). Horse A showed agglutination in 1:400, though the antitoxin content by the flocculation test was higher than that of the previous horse. Horses 140 and 141, on the other hand, showed the highest antitoxin content by the Ramon test, and at the same time did not give any agglutination in any dilution with the same bacterial antigen. These results would seem to indicate that the antitoxin content of the serum as manifested by the Ramon test is not dependent on the bacterial content of the serum for the reaction, contrary to the view propounded by Brofenbrenner and Reichert in their work on "The Nature of the Toxin-Antitoxin Phenomenon." Whatever the nature of the Ramon flocculation may be, the immediate object is to obtain a reaction between toxin and antitoxin for the purpose of determining the potency of one or the other. Substitution of an atoxic filtrate for toxin or of an antibacterial antigen for antitoxin as done by Bronfenbrenner and Reichert to prove their contention, does not constitute the Ramon test; it merely produces the inevitable bacterial precipitation reactions. These reactions, though useful for the determination of antigen-antibody content, are of no value for the quantitative determination of either toxin or antitoxin. Failure to obtain results by the Ramon test may be attributable to improper technic, such as dilution of toxin, too small amounts in the test tube for a clear-cut reaction, unsuitable temperature and, last but not least, wrong proportions between toxin and antitoxin.

Annotation: It is very essential to have a toxin of high potency. Because the scarlatinal toxins deteriorate rather rapidly, they should be used reasonably fresh. The deteriorated toxin may still be of use for intracutaneous tests in children, after it has lost its flocculating power. The sera also should be fresh when used.

STUDIES ON THE PARATYPHOID—ENTERITIDIS GROUP

VII. Enteric Infection ("Food Poisoning") Due to Tapioca Pudding Contaminated With B. Cholerae Suis (B. Suipestifer)

Published in The Journal of Medical Research, 1922, Vol. XLIII, pp. 53-59

Charles Krumwiede, Dorothy J. Provost and Georgia M. Cooper

On August 30th, 1921, there was delivered to us a sample of tapioca pudding and some uncooked tapioca. These samples were accompanied by a report indicating that the cooked pudding was the cause of "food poisoning" among the members of a family "O."

The history of this family outbreak was as follows:

The family consisted of: A. O., 20 years; J. O., 10 years (sister of A. O.); Mrs. O. J., 24 years (sister of A. O.); E. J., 3½ years (child of Mrs. O. J.); J. J., 5 weeks (child of Mrs. O. J.).

Inquiry indicated that some item of the evening meal of August 26th, 1921, 5.30 P. M. was the cause of the "poisoning." One item of the meal, see Table I, was tapioca pudding prepared from a package of tapioca, part of the contents of which package had been used previously with no ill effect. The pudding was started at 2 P. M. A quart of milk, grade B pasteurized, delivered that morning was opened and poured into a sauce pan and brought to a boil. To this was then added the washed tapioca. The mixture was then "boiled" in a double boiler for three-quarters of an hour. While still scalding hot, three eggs were beaten and mixed with the pudding. One of the eggs used was in the household, the other two were purchased at the time from a "chain" store together with the pork chops and frankfurters (see below) to be used for the evening meal. The pudding was then placed in an open dish till cool, then in the ice-box. It was characterized as "cold" when served. Whipped cream (cream, pasteurized, purchased that afternoon) was added when served.

The source of each of the food items was investigated and no evidence could be obtained that they were the cause of trouble in other families.

Mrs. J. maintained that all the materials were in good condition, also that she had sufficient ice in the ice-chest and that the ice-chest contained no remnants of food at the time. Food was usually purchased only as needed. The fact that only two eggs were purchased for the pudding (see above) corroborates this statement.

As noted above, the evening meal was eaten at 5.30 P. M. August 26th. The items partaken of by the different members of the family are shown in Table I.

TABLE 1.
FOOD ITEMS, THEIR CONSUMPTION

Name	Ill	Pork Chops, Fried	Frankfurters, Boiled and Fried	Potatoes, Mashed	Peas, Canned, Boiled with Fresh Carrots	Milk "B," Pasteurized	Tapioca Pudding
A. O.	Yes	Yes	No	Yes	Yes	Yes	2 oatmeal dishes
J. O.	No	Yes	Yes	Yes	Yes	Yes	None
Mrs. J.	Yes	Yes	Yes	Yes	Yes	Yes	1 dessert dish
E. J.	Yes	No	Yes	Yes	No	Yes	½ dessert dish
J. J.	Yes	(Wholly Breast Fed)					

Clinically the disease was characterized by vomiting, cramps, diarrhoea, fever and prostration. The incubation periods are given in Table II. One case (A. O.) died. An autopsy was performed by Medical Examiner Dr. Emanuel Martin. Congestion of all the internal organs was very marked. The congested intestinal tract showed no ulcerations, but the mesenteric lymph nodes were uniformly enlarged to the size of "navy" beans. Cultures from the spleen at autopsy yielded no growth. The cause of death was reported as undetermined, possibly "food poisoning." The organs were submitted for chemical analysis to Dr. Alexander O. Gettler, whose examination was negative.

As was noted in Table I, one of those partaking of the evening meal ate none of the tapioca pudding and she was not ill. The data as brought together in Table II strengthened the assumption that the tapioca was at fault, in spite of the fact that the infant did not eat it.

TABLE 2.

AMOUNT OF TAPIOCA PUDDING IN RELATION TO THE PERIOD OF INCUBATION AND OUTCOME OF THE DISEASE

Name	Amount of Pudding Eaten	Approximate Incubation Period	Outcome
A. O.	2 oatmeal dishes	6-9 hours	Died
Mrs. J.	1 dessert dish	20 hours	Recovery
E. J.	½ dessert dish	20 hours	"
J. J. (baby)	None	?*	"

* Illness began about twenty-four hours after onset of mother's illness.

Paratyphoid-like strains were isolated from the tapioca pudding and from the liver of A. O. These were tested with the agglutinuating sera specific for the various paratyphoid types. A few of these sera agglutinated the strains to some extent. Two hog cholera serums, however (Hog cholera Arkansas from Schütze, Lister Institute, Hog cholera isolated from hog by Ten Broeck 1919, obtained from Theobald Smith), agglutinated all the strains to the titre of the serum (titre 1:6400+). The indication on direct agglutinuation was therefore in favor of B. cholerae suis (suipestifer).

Feces from three cases were examined after convalescence but B. suipestifer was not found.

A strain from the tapioca pudding and one from the liver were selected to immunize rabbits for the production of agglutinating serums. The agglutination and the agglutinin-absorption tests with these antiserums show that the tapioca strains and the liver strains are antigenically identical and that these strains are likewise identical with the hog cholera strain employed as the type.

Discussion.—The fact that one and the same type of B. cholera suis or B. suipestifer was isolated from the tapioca pudding, and from the liver of the fatal case, is seemingly conclusive evidence that the illness was due to this bacterium. That this bacterium was not present in a living condition in the other foods as served seems shown by the fact that J. O., who ate of every other food item except the tapioca pudding, was not ill. That the symptoms were not due to "food poisoning" in the sense that there was considerable preformed toxin in the tapioca pudding is indicated by the following:—At the most, the bacteria had only about two hours in which to multiply; furthermore, when we received the pudding it was not very toxic for experimental animals even when injected intraperitoneally.

Considering the fact that the number of B. cholera suis or suipestifer organisms in the pudding was not great, it seems more likely that the symptoms were essentially due to an intestinal infection. If this is so, the infant was probably infected by the mother. This view seems justified in spite of the fact that the infant's blood gave no agglutination in dilutions of 1:20. That the illness of the child was only a coincidence cannot be excluded, but the development of a sudden gastro-intestinal upset in a breast infant, who had been doing well, seems a less likely alternative.

How the tapioca pudding was infected is a question. The previous use of the dry tapioca and the negative bacteriological examination is against the assumption that the tapioca was contaminated. Even if it were contaminated, the subsequent heating should have sufficed to kill the contamination. The milk used in the pudding is excluded as it was pasteurized and then boiled. The eggs would be a very unlikely source and furthermore the pudding was scalding hot, when they were added. The cream was pasteurized and is excluded by the fact that the bacterium was found in the pudding to which no cream was added.

Two pork products were purchased with the eggs used for the pudding. That Mrs. O. J. contaminated her hands in opening the packages and thus transfer the bacterium from one or the other of the pork products to the pudding is, at least, a reasonable assumption. That the frankfurters were not the source seems indicated by the methods of manufacture. However, contamination of the outside of the casings, after the heating, cannot be excluded. That either this occurred, or that the pork chops were infected, is apparently the alternative forced upon us to account for the contamination of the pudding. If either the frankfurters or chops were contaminated, the subsequent cooking rendered them innocuous, for the one member of the family who escaped infection ate of both these items.

The conclusions seem warranted:—That the tapioca pudding was infected with B. cholerae suis from pork products. That a limited amount of growth took place, so that each of those eating the pudding became ill. That the illness was due to an infection and not an intoxication, the infection giving rise to symptoms in one case, in from 6 to 9 hours. The last conclusion is not in accord with the view that the acute symptoms, developing after a short incubation, are primarily due to toxin preformed in the food.

We are indebted to Mr. W. B. Drennan and to Inspectors Samuel Goldman and Samuel Zusman of the Bureau of Food and Drugs for information and coöperation in obtaining the specimens for bacteriological examination.

REFERENCES

No references to the general subject of "food poisoning" seem necessary in this report. We have been able to find only one reference where tapioca pudding was apparently involved in a "food poisoning" outbreak. Sewall, Smith and Priestly (Jour. Roy. Army Med. Corps, 1920, 34, 510) report an extensive outbreak in a hospital. The authors consider the source of the infecting organism to have been the canned milk used in the pudding. Tea, to which this milk was added, was apparently a contributing agent in the outbreak. The strains isolated from some of the cases fell into the general group of "food poisoning" paratyphoid types. The agglutination and agglutinin absorptions were not sufficiently complete to definitely identify the type.

For cultural and serological separation and serological identification see:
Krumwiede, Kohn, and Valentine. Jour. Med. Res., 1918, 38, 89.
Krumwiede, Valentine, and Kohn. Jour. Med. Res., 1919, 39, 449.
Schütze. The Lancet, 1920, 1, 93.
Ten Broeck. Jour. Exp. Med., 1920, 32, 33.

STUDIES ON THE PARATYPHOID-ENTERITIDIS GROUP.

An Epidemic of Food Infection Due to a Paratyphoid Bacillus of Rodent Origin.[1]

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By Ole Salthe and Charles Krumwiede

On April 9, 1923, reports were made to the Bureau of Foods and Drugs that a number of persons were ill of "food poisoning." The reports were accompanied by a statement that this illness was due to the consumption of cream-filled crumb cake and eclairs. An investigation was immediately undertaken. This quickly showed that the food items mentioned were probably those at fault. They were the only common foods among the cases in the various families affected, and in each instance the purchases were made from the same bakery on the same date, April 5, 1923.

An investigation at the bakery showed that no stock had been carried from the previous day; that is, the illnesses in 53 cases brought together were associated with the baking of April 5th. Subsequently, six more cases of illness developed, as shown in Table 1. This table gives a summary of the cases, the items eaten, the baking involved and the approximate incubation periods, as indicated by the data obtainable.

The illnesses were characterized by the usual symptoms, nausea, vomiting, cramps, diarrhoea, some fever, headache and prostration. The severity was roughly proportionate to the amount of the infected food eaten. None of the infections was very severe and there were no deaths.

The bakery involved was a high class shop, clean, orderly and free of flies and vermin. The baking was done in the basement, which in point of cleanliness was above the average. No vermin was noted. The owner had three cats to prevent rodent invasion of the basement, which, according to his statement, was troublesome, if cats were not kept.

The personnel employed was cleanly, and none gave any history which could be interpreted as bearing on the outbreak. Several successive samples of feces, urine and blood were obtained from each individual in the bake shop and bakery; all were negative.

Our suspicion was immediately centered on the cream filling, because this would serve as a very good culture medium, if contaminated. The baked dough of the eclairs and crumb cake did not seem a likely source, for each individual piece would have had to be contaminated in the handling, subsequent to baking.

The cream filling was prepared by adding dried milk and sugar to water. This was then boiled. A mixture of cornstarch, eggs and water was then stirred into the above, the boiling being continued until a thorough mixture was obtained. The mixture was then poured into a wooden pail, and this

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[1] From the Bureau of Foods and Drugs, and the Bureau of Laboratories, Department of Health, New York City.

We are indebted to Inspectors E. M. Hoctor, Christopher Matthews, E. Bocker, J. Turner and J. Goechler, who collected the field data and obtained the materials for examination.

Miss Georgia M. Cooper, Miss Dorothy Provost, and Miss Marie Luckenbacker made the bacteriological examinations and carried out the serological work for identification.

TABLE I
SUMMARY OF CASES

Number of persons ill per family:	Food consumed:	Baking of†	Eaten	Illness began:
4.....	Eclairs	April 5, 1923	April 5, P.M.	April 6. P.M. to April 7, A.M.
2.....	"	" " "	" " "	April 6, A.M.
3.....	Crumb cake	" " "	" " "	April 6, early A.M.
4.....	" "	" " "	" " "	April 6, noon to April 7, A.M.
3.....	Eclairs	" " "	" " "	April 6. noon.
2.....	Crumb cake	" " "	" " "	April 6.
1.....	" "	" " "	" " —	April 6.
2.....	" "	" " "	" " —	April 6.
2.....	Eclairs	" " "	" " —	April 5, P.M.
1.....	"	" " "	" " —	April 5, P.M.
2.....	"	" " "	" " P.M.	April 5, P.M. to April 6, A.M.
1.....	"	" " "	" " "	April 6, A.M.
5.....	"	" " "	" " "	April 6, A.M.
4.....	Crumb cake	" " "	" 6 —	April 7, noon.
1.....	" "	" " "	" 5 P.M.	April 6, A.M.
2.....	" "	" " "	" " "	April 6, A.M.
1.....	" "	" " "	" " "	April 6, A.M.
4.....	" "	" " "	" " —	April 5, night.
3.....	Eclairs	" " "	" " —	April 6. A.M. to P.M.
4.....	"	" " "	" " —	April 6, A.M. to April 7.
2.....	?*	?*	?*	?*
3.....	Crumb Cake	April 9, 1923	April 9 —	April 11, A.M.
3.....	Eclairs	" " "	" 10 P.M.	April 10, night

* Home closed at time of inspector's visit; cases most probably connected with the April 5th baking.

†The pastry was prepared in early morning hours of the day noted.

stood on the floor, near the rear basement door. The mixture was usually stirred at intervals to accelerate cooling. Whether such stirring was actually done on the day in question could not be determined. The filling thus prepared was used for 120 eclairs, 50 cream puffs and 12 cream-filled crumb cakes.

Although the heating in the preparation of the cornstarch mixture would exclude the raw materials actually used, there remained the possibility that contaminated raw materials might find their way into the mixture after cooling. All open packages of raw materials were, therefore, embargoed and examined bacteriologically. All gave negative results.

The inspector, visiting each of the household involved, succeeded in obtaining left-over samples of both cake and eclairs of the April 5th baking. Bacteriological examination showed the presence of bacilli of the paratyphoid group. Because of other work which we were carrying out on the serological relationship of members of this group, we were able to make an immediate tentative identification of the bacilli present as *B. pestis caviae*. This identification was subsequently verified by a complete serological study of not only the agglutinative, but also the antigenic and absorptive characteristics. Stool specimens from 11 of the patients yielded, in 6 instances, bacilli of the same variety; they were shown to be like those in the cream in regard to their agglutinative, antigenic and agglutinin absorption characteristics. The details of the serological tests are not given here, but will be included in a later study on the serological classification of the paratyphoid group.

The results of the bacteriological examinations of the feces are tabulated (Table 2) since they have a practical value as indicating to some extent the persistence of the infecting type in the intestinal tract.

TABLE 2.

FECAL EXAMINATIONS

Case	Onset	Feces collected	Results as to presence of *B. pestis caviae*
D. P....	April 5th—night	April 11th	Positive
V. H....	April 5th—night	April 11th	Negative
S. M....	April 5th—night	April 11th	Positive
Mrs. F..	April 5th—night	April 11th	Positive
M. F....	April 5th—night	April 11th	Positive
M. J....	April 10th—night	April 17th	Negative
E. J.....	April 10th—night	April 17th	Negative
E. J.....	April 10th—A.M.	April 17th	Negative
H. B....	April 11th—A.M.	April 14th	Positive
F. J.....	April 11th—A.M.	April 14th	Negative
A. J.....	April 11th—A.M.	April 14th	Positive

The identification of the offending bacterium as *B. pestis caviae* gave us a definite clue on which to base a search for the source of contamination. Three possibilities immediately arose (see below, as to sources of *B. pestis caviae* type): bacterial rat viruses, rodents and the cats. The latter animals were removed to the laboratory and the feces (cage sweepings) examined seven times with negative results. No rat viruses had been used in the bakery or in any of the surrounding buildings. Traps were set and 11 mice, but no rats, were caught. Bacteriological examinations of the liver, spleen and intestinal contents of the mice were negative.

Before the above examination had progressed very far, a significant finding was made which apparently explained the mode of contamination of the filler. Besides a cleaning up of all utensils, etc., we insisted on the moving of all bulk supplies, and the shelves and floor were carefully inspected for the presence of rodent droppings. Rodent excreta were found on two boards used as storage shelves. Bacteriological examination showed that the excreta from the lower shelf were negative, but those from the upper one contained *B. pestis caviae*. This shelf was nearly over the place where the pail of filler was placed to cool.

How the rodent droppings got into the pail of filler is a question. Most probably this occurred through the agency of the cats jumping to the shelf in the endeavor to get at the mice or rats and thus scattered the feces into the pail.

The development of six subsequent cases, due to the baking of April 10th, was noted above (Table 1). An eclair was obtained from one of the families involved and stool specimens were also collected (Table 2). *B. pestis caviae* was isolated from the eclair and from two of the stools. This recurrence was evidently due to the same factors that gave rise to the first 53 cases.

The fact that only six cases developed from the baking of the 9th indicates that the contamination was slight and localized. This emphasizes a condition probably similar in regard to the baking of April 5th. As was noted in Table 1, 53 individuals in 21 families were made ill by the pastry of this day's baking. The total baking of this day consisted of 120 eclairs, 50 cream puffs and 12 crumb cakes. Ten of the families had eaten crumb cakes. This accounts, therefore, for at least 10 of the 12 crumb cakes prepared. Thirty-one persons ate eclairs. Even if a majority ate more than one apiece, over half of the number prepared is unaccounted for. No reports of illness due to the cream puffs were obtained. These facts indicate that the contamination of the crumb cakes was nearly uniform, that of the eclairs irregularly scattered, and that the puffs were either not contaminated or only to a negligible degree.

In the families visited, none who ate of the cream-filled cake or eclairs escaped at least some illness. It is difficult to understand how the irregularly contaminated eclairs happened by chance in the sales to go to the families in this way. This is especially true, as we know of instances, and there must have been many others, where eclairs were eaten without ill effect.

The method of filling the pastry may explain the peculiar distribution of the contamination.

The contamination must have been on the surface of the cream mixture, as it was cooling. If stirred this would tend to spread the contaminant. The following facts indicate that such stirring only served to spread the contamination in the upper layers. When cooled the mixture was poured into a filler, from which the eclairs were filled. Presumably the upper contaminated portion went into some of the eclairs which were filled first. The subsequent pourings into the filler were probably not contaminated. This would explain the absence of illness due to some of the eclairs and the cream puffs, which were filled after the eclairs.

The crumb cakes were the last item prepared. The cream was spread with a spatulum. Most probably the contaminated cream adherent to the side of the pail was scraped off, mixed with the remaining cream mixture in the bottom of the pail and then spread on the cakes. This procedure would explain the reason why nearly all of the crumb cakes were reported to have caused illness.

Undoubtedly multiplication of the contaminating bacteria played a considerable rôle in raising the infectiousness of the individual pastry items. As we pointed out in a recent report, this must be considered as a probably important factor, even where the time is short between preparation and ingestion. In the present epidemic a considerable time intervened in most of the cases (Table 1).

We have designated the causative strain as *B. pestis caviae*, because, among other reasons, in a previous study we had utilized a guinea pig strain as a type strain. In this work we were able to show by agglutination and agglutinin absorption that many of the paratyphoid strains, prevalent in rodent infections, were serologically alike and differed distinctly from the other paratyphoid types which had been differentiated up to that time. Some of the so-called strains of *B. typhi murium* from mice were found to be like *B. pestis caviae*. The majority, but not all the strains (exclusive of *B. enteritidis*) of guinea pig or mouse origin, were of this type. A strain from a rabbit and also one from a cat were likewise found to be of this type. The last was the reason for our attention to the cats in the bakery (see above). Since our earlier work, we have identified, as of this type, strains from hogs, a calf, a cow, a swell as strains from several avian species, also several strains from human sources. These will be discussed later. This would indicate that, if the type is primarily a rodent pathogen, it possesses a definite and well-marked ability to cause disease in many other species.

Schütze, in his serological study of paratyphoid strains, has added considerable data in this regard. Among the cultures studied, he separated a group which he designated as the "mutton type." Seven food infection strains, a strain obtained from one of sporadic gastro-enteritis cases, a strain obtained from a guinea pig epidemic, a strain from an epidemic among calves, one laboratory strain labelled psittacosis, presumably of avian origin, and other laboratory strains of unknown origin, fell into this group.

Through the courtesy of Dr. Schütze we obtained his "mutton" strain and it is identical with the strains we have designated as *B. pestis caviae*.

Undoubtedly, if one were to review carefully the serological data available in the various epidemics traced to paratyphoid types (exclusive of *B. enteritidis*), one could develop a presumption that a considerable number were due to the *B. pestis caviae* type, here described. Uhlenhuth and Hübener in their description of *B. typhi morium* cite a series of food infections which presumably were associated with the use of "rodent viruses." Were these viruses at fault and were it definitely shown that *B. enteritidis* was not contained in the viruses, there would be at least a presumption that the infections were due to the *B. pestis caviae* type.

As there is no certainty involved in these outbreaks, no summary of them is made. Now, that the prevalence of a definite serological type is recognized and available for comparative study, the relative incidence of *B. pestis caviae* can be established with certainty in future outbreaks.

As far as we know, the following is all the information available as to the incidence of this type in human infections on this continent. We have a culture of this type which according to the data accompanying it was isolated by Weinzirl in connection with food infection due to milk. We have been unable to find any published data, and do not know in which state this outbreak occurred.

The outbreak of food infection reported by Bernstein and Fish was caused by this paratyphoid type. Pie was the vehicle, and the culture was obtained from a cocoanut-custard mixture. We have the culture from this epidemic and have identified it as *B. pestis caviae*.

Winslow, Hiscock, Rogers and Robinson have recently reported an epidemic of food infection due to an egg salad. The contamination was seemingly of the mayonnaise dressing. They isolated the offending bacterium from the stools of some of the cases, and, on the basis of the cultural agglutination and agglutinin absorption reactions, termed the bacillus *B. paratyphosus B.* The agglutinations were carried out with an antiserum for a case strain and one for *B. paratyphosus B.* (Schottmüller).

Through the kindness of Dr. Winslow we have been able to add some of these strains to our collection and to test them with our various type antiserums. Our tests leave no doubt that the bacterium is a *B. pestis caviae* type and not a *B. paratyphosus B.* That is, it is different from Schottmüller type, although generically it might be considered a member of a *B. paratyphosus B.* group. It would take far too much space to go into the technicalities of agglutinin absorption and into the subject of the peculiar reciprocal reactions which occur within the paratyphoid group. This would be necessary, if we were to discuss the agglutinin absorption reactions which Winslow and his co-workers record. Our rather extensive observations on these subjects will be published shortly.

Winslow and his co-workers considered the cook who prepared the mayonnaise as the most probable source of the contamination. This would be the natural conclusion, especially if the offending bacterium were considered to be a *B. paratyphosus B.*, in the usual sense.

The rôle of human beings as persistent carriers of the *B. pestis caviae* type is still an open question. The data which we possess indicate that human beings may become carriers. In the case of the more severe types of food infection, at least, some bacteriaemia probably develops and biliary localization is a possibility.

We have identified as *B. pestis caviae* cultures from persons suffering from other conditions than food poisoning. Thus a strain sent to us, labelled as having been isolated by Wherry from the liver of a three-year-old boy with status lymphaticus, is of this type. We have isolated this type from the stool of a woman showing some fever of obscure origin. Her history indicated the existence of a chronic cholecystitis. Two cultures sent us from Panama are also of this type. The clinical history we have thus far obtained suggests that the two cases had a febrile condition of typhoidal character. Both gave "positive" blood cultures. These cases indicate the ability of this bacterium to invade the body and to cause more generalized infections, some of which might terminate in a carrier condition.

Our reason for continuing the use of the term *B. pestis caviae*, as suggested by Theobald Smith, in preference to *B. typhi murium*, is that this description of the guinea pig organism indicated that it was probably a variety distinct from the other paratyphoid types known at that time. Most of the German workers, however, have contended that *B. typhi murium* was essentially a *B. paratyphosus B.* type. Schütze's term "mutton type" is, as he frankly admits, a temporary expedient waiting a better terminology. It does not seem necessary to review the earlier serological work on the strains of rodent origin. The methods, as seen from the standpoint of to-day, were very inadequate and the deductions contradictory. The essential result was not arrived at, namely, that there was a definite and distinct paratyphoid type prevalent among the guinea pig and mouse strains.

In the event of an outbreak, such as we have described, a public administrator is placed between the upper millstone of justice to the storekeeper and the nether millstone of protection of the public. When we were convinced that the bakery shop in question was at fault we faced this dilemma. We did not deem it wise to close the bakery, as such an act is disastrous to the storekeeper, even if it does not bankrupt him. The fact that none but the cream-filled products were involved, and that the bakings of the 6th, 7th and 8th had been eaten without ill effect, indicated that a thorough cleanup and scalding process with a method of protection of the filling during cooling would suffice. Furthermore, nothing had occurred in the preceding years' bakings which were made under the same circumstances. We appreciated, however, that the most real protection was to discover the source of the contamination.

The report of six cases of illness due to the baking of the 9th, a baking which preceded our cleanup, was a source of uneasiness, however. The persistent publicity given the affair by the newspapers, urged on by a publicity agent and the mouth-to-mouth neighborhood gossip, centered attention on the bakery, with the result that the baker, a highly conscientious man, voluntarily discontinued the manufacture of the "cream"-filled products.

After we felt sure of the source of the contamination, we urged him to resume production with precautions to prevent contamination. This he was loath to do, so we arranged with him to prepare the filling on several successive days. We took the finished product to the laboratory, examined it directly and, after incubation, with negative results. He then resumed commercial production and no untoward results have occurred to date, a period of nearly four months.

Summary

The food infection outbreak, here reported, comprised 59 cases. The cause of the outbreak was the contamination of the cream (corn-starch) filling of eclairs and crumb cake. The contamination was found to have been of rodent origin. The causative bacterium was a distinct paratyphoid type, *B. pestis caviae* (*B. typhi murium*—"mutton" type), prevalent in rodents; it has also been isolated from other species. This type, only recently separated with definiteness as a distinct and prevalent paratyphoid variety, is apparently one of the commonest etiological agents of food infection in man.

The *B. pestis caviae* (*B. typhi murium*—"mutton" type), *B. cholerae suis* and *B. enteritidis* are apparently the three varieties most likely to be expected in cases of food infection. One gains the impression that, had the *B. pestis caviae* type been recognized earlier as a distinct paratyphoid type, it might have been found to be the most common of the three varieties.

For bibliography: see the original article.

EFFECT OF STORAGE AND CHANGING SEA WATER ON CONTAMINATED OYSTERS

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In the February issue of the Journal, our experiments on the effect of chlorination on oysters contaminated with typhoid bacilli were reported. Through the exigencies of time and available Journal space the collateral experiments on the effect of changing sea water alone were not presented. These as well as observations on the effect of dry storage were held for report at this time.

When the increase of typhoid fever occurred during the latter part of 1924, it was noticed coincidently in inland as well as seaboard cities. When investigations showed that this increase of typhoid fever was attributable to contaminated oysters it indicated that *B. typhosus* must persist in dry, stored oysters for some time. Experiments were undertaken, therefore, to determine the time of survival of *B. typhosus* under storage conditions.

Oysters for these observations were dredged at West Sayville, Long Island, and sent immediately to the laboratory. The water was obtained from Great South Bay, Long Island.

Two lots of about 80 oysters were contaminated by suspending the oysters in wire crates in tanks containing about 8 gallons of contaminated sea water: Lot A, shown in Table I, in sea water contaminated by the addition of a suspension of feces containing *B. Typhosus;* Lot B, shown in Table 2, in water contaminated with freshly isolated cultures of *B. typhosus*. The tanks were kept overnight at room temperature (18° to 20° C.). The oysters drank actively. They were then removed from the tank for storage. The storage conditions approximated in temperature that of the colder

TABLE 1.

SURVIVAL OF B. TYPHOSUS ON OR IN OYSTERS, CONTAMINATED WITH FECES, DURING STORAGE. FIRST EXAMINATION FEBRUARY 4, 1925

	Estimated number of B. typhosus per		
Days of Storage	cc. of Liquor	cc. of Body Emulsion	Shell
0	254	Not exam.	Not exam.
1	62	110	Not exam.
5	23	10	Not exam.
7	10.4	0.9	3,111
9	20.4	1.85	194
10	2.5	+(<0.08)	142
14	2.5	1.6	Neg.
21	7.6	1.5	Neg.
28	1.3	+(<0.08)	Neg.
35	30.6	0.25	Neg.
41	1.5	+(<0.08)	Neg.
49	0.25	0.25	Neg.

TABLE 2.

SURVIVAL OF B. TYPHOSUS ON OR IN OYSTERS (CONTAMINATED WITH FRESHLY ISOLATED CULTURES OF B. TYPHOSUS) DURING STORAGE. FIRST EXAMINATION FEBRUARY 4, 1925

Days of Storage	Estimated number of B. typhosus per cc. of liquor	cc. of Body Emulsion	Shell
0	37,700	2,118	Not exam.
1	13,870	1,260	Not exam.
5	10,333	1,077	Not exam.
7	7,107	450	Not exam.
9	4,514	473	2,733
14	1,245	515	2,750
21	5	100	Neg.
28	1.6	0.6	100
35	1.4	12	Neg.[1]
41	103	32	Neg.
49[2]	45	26	Neg.

[1] Oysters removed on this date and placed in fresh sea water then showed 160 *B. typhosus* per shell, irregular distribution.

[2] Oysters dead.

months (February and March) of the oyster season. The oysters were kept out-of-doors in a small shed on the north side of a building which was covered with snow. When snow was no longer available they were transferred to an icebox.

The materials used for bacteriological examination were: washings from the shell, using 10 cc. of broth per oyster, the removal of the contaminated material being assisted by rubbing with cotton swab; the liquor; and the body of the oyster emulsified in 5 cc. of broth. The number of *B. typhosus* was estimated from the number of colonies on the plates or from the smallest quantity which, when placed in an enriching brilliant green broth, yielded *B. typhosus* on subsequent plating.

The effect of dry storage on the numbers of *B. typhosus* on or in contaminated oysters is summarized in Tables 1 and 2. The number of typhoid bacilli as shown in Tables 1 and 2 were greatly reduced, but bacilli still could be isolated from both liquor and body after 49 days of storage. Before this the oysters had begun to die and those examined on the last day of the experiment were unfit for food. The number of *B. typhosus* per shell was very low after 2 to 4 weeks' storage; the shells of the more lightly contaminated oysters being negative after 2 weeks, whereas with the more heavily contaminated ones the last positive finding was after 5 weeks' storage.

A parallel series of observations on the persistence of *B. coli* was also carried out using the presumptive broth fermentation tube method. The results of these observations are illustrated in Table 3. In this table as in Tables 1 and 2 all of the examinations are not recorded but only a sufficient number to show clearly the rate of decline in numbers of *B. typhosus* or *B. coli.* In the observations on the survival of *B. coli* only one lactose broth tube was used for each dilution tested. Evidently the actual numbers may have been the estimated numbers given or somewhere between this figure and one-tenth of this figure. Although the contamination with *B. coli* was very heavy and considerable irregularities are noticeable in the results in Table 3, there is a general parallelism in the rate of reduction of *B. typhosus* and *B. coli* on dry storage.

TABLE 3.

SURVIVAL OF B. COLI IN OYSTERS, CONTAMINATED WITH B. COLI, DURING STORAGE. FIRST EXAMINATION FEBRUARY 4, 1925

Days of Storage	Estimated number of B. coli per c.c. Liquor	c.c. Oyster Body Emulsion
0	82,000,000	200,000
1	425,000,000	25,500,000
6	183,000,000	250,000
8	685,000	25,000
10	550	50
21	5,050	505
28	50	5,050
35	50	550
41	1	5.5
49[1]	10	1

[1] Oysters dead.

Another observation on the survival of *B. typhosus* in dry stored oysters was made with some oysters which had been subjected to chlorination. Before chlorination (less than 1.5 p.p.m. of chlorine) the number of *B. typhosus* was 103 per cc. of liquor and 19 per cc. of oyster body emulsion. After storage for 8 weeks (refrigerator) 1 cc. of liquor contained 1.2 typhoid bacilli and 1 cc. of oyster body emulsion gave 0.19 *B. typhosus.*

These experiments show that *B. typhosus* may persist in oysters for a period of time beyond any time of storage which occurs in the trade.

The death of oysters during the period of storage led naturally to the question as to whether a dead oyster was a favorable culture medium for the typhoid bacillus. Killed contaminated oysters were stored at room and icebox temperature and approximately equal portions of body substance were ground in 2 cc. of broth and culture. In oysters contaminated with pure cultures and held at room temperature there was evidence of a slow multiplication approximating 15 times after 7 days, followed by a more rapid rise to approximately 1,000 times after 10 days. At icebox temperature there was a moderate increase to the 7th day, then a slight decrease, the number being approximately the same on the 10th day as on the 1st. In oysters contaminated with stool suspensions, at room temperature the overgrowth of other organisms prevented the isolation of *B. typhosus* by the direct plating method after the 1st day. By enrichment methods *B. typhosus* was recovered up to the 10th day. At icebox temperature *B. typhosus* was isolated up to the 10th day, the last examination made. There was no noticeable increase during this time. These observations indicate the possibility of recontamination by the drip from dead oysters, which contamination would be greater if the temperature were such as to allow growth.

As was to be expected, other workers were impressed by the occurrence of oyster-borne typhoid fever in inland cities and started to investigate the viability of *B. typhosus* on or in oysters.

Kinyoun reports that oysters heavily contaminated with stock cultures of *B. typhosus* yielded *B. typhosus* up to the 15th day, when the observations were terminated.

Jordan likewise contaminated oysters with culture strains of *B. typhosus* and stored the oysters at 5° to 8° C. He isolated *B. typhosus* from the interior of the oyster up to the 24th day. After this the results were negative and the experiment was terminated on the 30th day.

Tonney and White also contaminated oysters with stock cultures of *B. typhosus.* The contamination was extremely heavy. The results with those stored at 45° F. only are quoted. The oysters were opened in the ordinary way, up to the 17th day, so that the typhoid bacilli isolated up to this day may have been from the shell or from the liquor. After the 22nd day the liquor was collected in a way to avoid contamination of the shell. In the case of dead oysters washings were examined. The results were positive to the 60th day, negative thereafter. The oysters remaining after the 39th day were all dead.

Of the earlier observers, Field working in this laboratory showed the persistence of *B. typhosus* for the longest time, *viz.*, 42 days, when the experiment was terminated.

The question of the rate of cleansing which occurs when contaminated oysters drink in successive changes of sea water has been the subject of earlier investigations. We undertook a reinvestigation of this subject because we felt that the means of isolation of *B. typhosus* at our disposal would possibly demonstrate a longer persistence of *B. typhosus* than was indicated by earlier observations. This has been the case, but the conditions of the experiment were not completely satisfactory for the longevity of the oyster and death of the oysters terminated the observations.

The 50 oysters for this experiment were contaminated with typhoid feces and then placed in 4 gallons of fresh sea water. The water was changed 18 times in a period of 24 days. The temperature in the shed where the tank was kept was from 60° to 70° C. and just prior to each change of water samples were taken for examination. Table 4 gives the results of a sufficient number of these observations to indicate the rate of decline in the number of *B. typhosus* present.

TABLE 4.

EFFECT OF CHANGING SEA WATER ON THE NUMBER OF B. TYPHOSUS ON OR IN OYSTERS

Days after contamination	No. of changes of sea water	Estimated number of B. typhosus per cc. Liquor	Estimated number of B. typhosus per cc. Oyster body emulsion	Estimated number of B. typhosus per shell	B. typhosus per cc. of sea water in tank
0	0	2,675	213	40,400	12,300
1	1	424	68	9,500	550
3	2	64	42	980	800
4	3	2	0.5	75	Neg.[1]
7	6	8.6	0.5	64	Neg.
11[3]	9	4.	+<0.1	84	Neg.[2]
15[4]	12	7	1.5	74	0.75
21	16	1	2.2	1.2	0.25
24[5]	18	1.6	2.2	3.7	Neg.

[1] Negative in this column means *B. typhosus* not isolated from 20 cc. of water.

[2] Positive on the 8th and 10th days, 7th and 8th change.

[3] Still drinking, grayish slime developing on shells.

[4] Same condition, one dead oyster found.

[5] Remaining oysters dead, slime increased, water had disagreeable odor.

The results indicate that 3 successive drinkings in fresh sea water will result in a reduction of about 99 per cent. of the typhoid bacilli on or in contaminated oysters. This degree of reduction compares favorably with that which was obtained (see previous number of this Journal) with successive treatments with chlorinated sea water and intermediate drinking. There was a difference however; with simple changes of plain sea water *B. typhosus* were isolated from the tank water almost to the end of the experiment. This continued contamination was evidently due to the rinse from contaminated oyster shells and the excretion of contaminating *B. typhosus* from within the oysters. With chlorination, however, we usually failed to isolate *B. typhosus* from the tank water. The exceptions were with heavy contamination in which case the chlorine added was unable to destroy all the typhoid bacilli in the tank water.

The failure to keep oysters alive for a sufficient time in the previous experiment was apparently due to eventual fouling of the water and reduced oxidation of the sea water which was stored in barrels. Permits are granted during the closed season for the removal of oysters from condemned beds to uncontaminated waters for cleansing. It was the intent of the above experiment to give some indication in this regard. Although the indication is that even 3 weeks is insufficient, it seemed desirable to repeat this experiment under conditions more favorable to the oysters' activity. As cold weather had supervened this could not be done, but we plan to carry out such an experiment as soon as the natural waters have warmed sufficiently to insure active drinking.

In the meantime we have investigated the length of time that *B. typhosus* will survive in oysters during the winter months when the oysters are returned to natural waters. The place selected for this experiment was in a bay near a series of condemned oyster beds. The oysters for the experiment were secured from these beds so that the factor of change to different waters might not enter. These oysters were contaminated with typhoid feces. The contamination was comparatively light, 100 *B. typhosus* per shell, 3 per cc. of liquor and less than 0.4 per cc. of body emulsion. The oysters were submerged in the bay water in lots in wire crates and then brought to the laboratory at intervals for examinations.* The results of these examinations are given in Table 5.

TABLE 5

PERSISTENCE OF B. TYPHOSUS IN CONTAMINATED OYSTERS RETURNED TO NATURAL WATERS, 10 OYSTERS EXAMINED AT EACH TEST

No. of days in bay	Date	Temperature of water	Number positive for B. typhosus Shells	 Liquors	 Bodies	Pooled Gills[1]
0	12-2	....	10	10	9	Pos.
1	12-5	Not taken	0	1	3	Pos.
4	12-9	43° F.	0	1	1	Pos.
10	12-14	36° F.	0	1	1	Pos.
17	12-21	41° F.	0	0	1	Neg.
24[2]	12-28[2]	39° F.	0	0	1	Neg.
31[2]	1-4[2]	39° F.	0	0	2	Neg.
39	1-12	30° F.	0	0	0	Neg
51	1-26	36° F.	0	0	0	Pos.

[1] Gills from the 10 oysters put in brilliant green broth.

[2] Five control oysters (not contaminated but placed in same waters) examined on each date gave negative results.

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*We are indebted to Dr. Rowland G. Freeman for taking care of these details.

The temperature of the water was taken as noted. As a further guide to the probable temperature of the water during the intermediate times we quote the following from the Weather Bureau report:

MEAN DAILY TEMPERATURE (FAHRENHEIT)

December, 1925

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
38°	43°	44°	48°	50°	50°	43°	40°	36°	30°	32°	41°	40°	32°	31°	32°
17	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	
32°	31°	34°	41°	40°	37°	28°	32°	32°	20°	10°	23°	28°	26°	26°	

January, 1926

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
33°	38°	39°	42°	42°	44°	30°	25°	27°	28°	28°	28°	20°	24°	32°	34°
17	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	
34°	46°	44°	42°	46°	30°	22°	26°	23°	28°	32°	22°	14°	30°	39°	

It is probable from the data given that little drinking took place. These observations indicate therefore that should oysters become contaminated during their more inactive period, *B. typhosus* can persist in them for at least 51 days. It was a surprise to us that we should succeed in isolating *B. typhosus* after this length of time considering how light the initial contamination was. The length of time of persistence was equally surprising as there was no reason to believe that *B. typhosus* would survive for this time even in inactive oysters. On the other hand, the conditions might be considered as comparable to dry storage because of the inactivity, and *B. typhosus* will persist for this time in dry stored oysters.

It might be argued that the bacilli isolated came from the relatively unsafe waters in which the oysters were placed. This we believe was not the case as shown by the control (uncontaminated) oysters. The numbers of control oysters were small due to accidental loss of some crates through breaking of the tie-ropes. Even so, we believe that the results indicate that the *B. typhosus* isolated were from the artificial contamination.

Some of these oysters were examined for *B. coli*. A score of *zero* was obtained on oysters yielding *B. typhosus*. It has not infrequently been noted in our experiments that *B. coli* colonies were very few in number or apparently absent on plate media which showed the presence of colonies of *B. typhosus*. Such observations emphasize what one tends to forget—a high *B. coli* score is an indication of danger, a low *B. coli* score is not necessarily an index of safety.

TABLE 6.

POSITIVE FINDINGS FROM ENRICHMENT BROTH (40 SAMPLES) ACCORDING TO DAY OF POSITIVE RESULTS

Days of incubation of brilliant green broth	Specimens grouped according to days of positive findings				
1	12+	28—			
3		10+	18—		
5			10+	8—	
7				6+	2—
10[1]					2—

[1] Observations continued until the 21st day but no more samples became positive.

In the above work and in that previously reported both the Endo medium and brilliant green agar (Krumwiede) were employed. As was to be expected, the latter frequently yielded positive results, where the former was negative. In addition, a brilliant green broth was used for enrichment. As we knew that the oyster liquor and especially the oyster body emulsion would reduce the activity of the dye, preliminary tests of this factor were carried out. As it would not be feasible to use several dilutions of dye with each of the many samples so as to strike the optimum in each instance, an average optimum was selected. For culturing the oyster body emulsion 1 cc. of a 1 to 1000 solution of brilliant green was added to 50 cc. of broth. For the liquor, 0.25 cc. of the dye solution was employed. The washings from the shell were cultured in 50 cc. of broth to which 0.25 to 0.5 cc. of dye solution (1 to 1000) was added, the amount varying with the quantity of dirt washed from the shells. Veal broth of a reaction of pH.7.2 was employed.

The tables have shown how efficient this procedure has been in isolating the residual few *B. typhosus*. The maximum efficiency of the procedure was not reached, however, till the latter half of our work, that is, until we found how successful repeated platings from the enrichment broth might be. To illustrate this we have tabulated the results of a series of successful enrichments according to the day on which positive results were obtained (Table 6). The 40 positive results are those obtained in 82 attempts at enrichments of *B. typhosus* in the last chlorination experiment we carried out. The table shows how the number of positive results increased on each succeeding examination of the broth.

SUMMARY

B. typhosus persisted in shell oysters stored at refrigerator temperatures for as long as the oysters lived, a period of 41 days. The oysters dying after this time were examined on the 49th day of storage and also yielded *B. typhosus*. The *B. typhosus* on the shells of stored oysters were apparently dead after 14 to 40 days, the length of persistence depending on the degree of contamination. A dead oyster serves as a culture medium and the number of *B. typhosus* will increase if the temperature is satisfactory for multiplication. Repeated changes of fresh sea water resulted in a rapid diminution of the number of *B. typhosus* on or in oysters, the diminution reaching about 99 per cent. after 3 changes of water. *B. typhosus* was isolated up to 21 days, during which time the water was changed 16 times, after which the remaining oysters died. The degree of purification following 3 changes of fresh sea water approximated that observed with a similar number of treatments of chlorinated sea water. There is a difference, however; in most instances the *B. typhosus* in the chlorinated water was apparently killed. Contaminated oysters were placed in natural waters the temperature of which was low enough to largely inhibit drinking. The oysters contained *B. typhosus* after 51 days in these waters, the last observation made. In some of these experiments as well as in some of those described in our previous report, note should be made of the prolonged persistence of minimal numbers of *B. typhosus* under different conditions. This prolonged persistence emphasizes the fact that apparently the only safe oyster is one which has been protected from any contamination with fecal pathogens for at least some months prior to harvesting. The actual time can only be determined by further careful experiments.

For Bibliography: See the original article.

THE CHLORINE TREATMENT OF CONTAMINATED OYSTERS

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The great success which has attended the use of chlorine for the purification of water supplies has led to an attempt to apply a similar procedure to the purification of contaminated oysters. Wells, on the basis of experimental observations, has advocated the use of chlorinated sea water treatment of oysters and believes that contaminated oysters thus treated may be rendered safe for consumption.

The following quotation from Wells (1920) indicates the mechanism on which he bases his procedures:

> Briefly stated, this method of purification consists of nothing more than assuring conditions of cleanliness under which the oyster can, by its natural function, remove any pollution received from the water. Ordinarily the oyster is very active in filtering out and digesting fine particles which are drifting in the water. If kept in clean surroundings, the oyster is just as active in returning these substances into the water. Under ordinary conditions an oyster passes 50 gallons of water a day through his gills, and a particle of food deposited on those gills will pass on to the mouth and be eliminated from the oyster within 5 hours. If conditions are maintained such that pollutions are removed, and new ones are not permitted to enter the oyster, it is possible to cleanse a polluted oyster within a remarkably short period. Twenty-four hours has been found sufficient under ordinary conditions of practice. With slightly polluted oysters less time is required than for grossly polluted oysters. The latter, which it would not be proposed to treat, might require a longer period than 24 hours.

From this standpoint the apparent value of chlorination rests on its efficiency in sterilizing the available water, the exterior of the oyster shells and the material excreted from the oyster. Indirectly it depends on the completeness of the excretion of contaminating organisms by the oyster itself. For a consideration of these aspects the experiments given below were carried out.

The following is an outline of the source of materials and the methods employed in our experimental tests of the efficiency of the chlorine treatment of oysters.

The oysters were specially dredged for us through the courtesy of one of the local oyster concerns.* They came from Great South Bay, Long Island. This insured fresh active oysters and the relative purity of the waters from which they were dredged helped to reduce the frequency and numbers of bacteria which would interfere with the isolation of specific types. The oysters were "reasonably clean." The water also was obtained from Great South Bay.

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* We are indebted to Mr. Hugh Taylor, Director of the Bureau of Foods and Drugs, and to Mr. Richard Ross of the same Bureau for arranging these details.

The oysters were contaminated on arrival at the laboratory by immersing them and allowing them to drink in sea water contaminated with feces containing *B. typhosus.* This procedure seemed more desirable than the use of culture strains. It approximates the natural conditions of contamination and leaves no question as to acquired or lessened resistance through cultivation. The oysters always drank freely and sedimentation resulted in the contamination of the shells.

For chlorination a concentrated solution of chlorine gas in tap water was employed. This was added in appropriate quantities to sea water to give the desired chlorine content. The oyster sea-water ratio was adjusted to within the recommendations quoted above. The details as to amount of chlorine, time of treatment, etc., are given with each of the experiments. The content of chlorine was determined by titration.

The oysters after contamination or treatment were prepared for bacteriological examination with the following possibilities in mind: Were *B. typhosus* present on the shell, in the liquor or, as far as could be determined, were they in the body of the oyster. Three sets of materials were therefore examined, washings from the shell, the oyster liquor and an emulsion of the body in broth. The first was obtained by scrubbing the shell with a cotton swab, in the later experiments with a tooth-brush using a constant quantity (15 cc.) of broth for each oyster. This served as an estimate of the degree of shell contamination. The oyster was then dipped momentarily in boiling water and drained, then opened and the liquor collected. The gills were then cut from the body and the body ground in a mortar with 5 cc. of broth. The volume of the oyster body emulsion averaged about 10 cc. Naturally this emulsion would contain traces of liquor. Comparative observations with washed and unwashed bodies and bodies treated with strong chlorine solutions indicate that the bacilli obtained from the body emulsion were mostly either in the mucus covering the body or in its interior. Actually this point is only of relatively academic interest, the practical point being whether *B. typhosus* were present or not.

The bacteriological examinations were planned to take advantage of the newer refined methods of isolating *B. typhosus,* thus making it possible to discover *B. typhosus* in many instances where the findings would have been negative with the older methods. The washings from the shell, the liquor and the ground oyster body suspension were separately inoculated in measured amounts and spread on brilliant green agar plates. From 0.1 to 0.4 cc. of the undiluted samples generally were used per plate, but where the necessity was indicated, dilutions were employed and 0.01 or 0.001 per plate inoculated; 6 to 10 plates were employed for each sample. After incubation the number of *B. typhosus* colonies per plate were counted. The identification was based on the results of inspection supported by slide agglutination and in doubtful cases confirmed by fishing to triple sugar medium and making further agglutination tests. Complete serological tests were made wherever the results were still indefinite. In addition to the direct plating of the material, the remainder of the sample was inoculated for enrichment into brilliant green broth. The broth was plated on brilliant green media at 24 hours and on later days, in some instances up to 3 weeks. The first positive findings of the broth were found on the 1st up to the 10th day inclusive. The necessity of repeated examinations is very apparent.

The figures given in the tables are based on the colonies per plate with correction for the volume employed or on an amount which yielded *B. typhosus* when added to brilliant green broth.

The number of oysters examined during or at the end of the chlorination experiments has varied. In the earlier tests only 2 oysters were examined at each test period. This resulted in some apparent discrepancies due to the unevenness of the distribution of *B. typhosus*, and the number examined was increased to 4. In the last experiment 9 oysters were examined at each time.

Chlorinated water was tried at different temperatures in some of the experiments to determine whether this would influence the effect of the chlorine and to determine the differences in the rate of chlorine fall.

The experiments are given below in the order in which they were carried out, in most instances with an accompanying table giving the details of the test.

Experiment 1 was a relative failure in that an excessive overgrowth of *B. aerogenes* types prevented the isolation of *B. typhosus*, in all the earlier tests in the series. The experiment is significant, however, in that with a treatment in chlorinated water (total available 36.3 parts per million at the start) we were able to isolate *B. typhosus* from the shell washings at the end of 27 hours in the bath. During this time the total chlorine had fallen from 36.3 to 1.2 parts per million. The temperature of the bath was from 9° to 14° C. during this period.

In chlorination experiment 2 (Table I) the contamination of the shell was heavy, 12,300 *B. typhosus* per shell, but the chlorine content was high, 32 parts per million. The test at the end of an hour showed a sharp fall in the number of typhoid bacilli, partly due to the wash effect of the chlorinated water removing the sediment from the shells. At the end of 5 hours there was no marked further decline in numbers, indicating that many of the bacilli were protected from the action of the chlorine. At the end of 24 hours in the cold tank the chlorine had dropped from 32 parts per million to 1.5 parts and in the warmer tank from 32 parts to 0.5 parts and typhoid bacilli were isolated in considerable numbers from all the shells tested. It

TABLE 1.

EFFECT OF CHLORINE ON THE NUMBERS OF B. TYPHOSUS ON THE SHELLS OF CONTAMINATED OYSTERS

Tank	Time of Treatment	Temp.	Chlorine p.p.m.	B. typhosus per shell
A	0	20° C.	32.1	12,300
	2 hrs.	13° C.	12.6	450
	3 hrs.	12° C.	8.9	570
	4 hrs.	12° C.	6.8	150
	24 hrs.[3]	6° C.	1.6[1]	60
B	0	20° C.	32.1	12,300
	2 hrs.	20° C.	11.8	2,800
	3 hrs.	20° C.	7.9	130
	4 hrs.	21° C.	5.3	160
	24 hrs.[3]	25° C.	0.5[2]	70

[1] In a control tank without oysters the chlorine was 21.6 p.p.m.

[2] In a control tank without oysters the chlorine was 10.2 p.p.m.

[3] Drinking probably took place some time before the 24th hour.

NOTE—Several examinations of the chlorinated sea water were made after the contaminated oysters were placed in the tank. These gave negative results.

should be noted, however, that the chlorine content fell between the 5th and 24th hour to a point where drinking and recontamination of the shells by excreted *B. typhosus* could have occurred. Deductions as to effect of the chlorine on the initial shell contamination must take this into consideration.

In view of the unfavorable results of the second experiment the experiment 3 (Table 2) was carried out with a relatively light contamination. The concentration of chlorine was approximately the same as the previous experiment. Typhoid bacilli were recovered from the shells of the oysters in both tanks at the end of 5 hours. At the end of 26 hours in the case of the tank kept at lower temperature no *B. typhosus* was recovered. It should be noted how the chlorine persisted in the latter tank. In the warmer tank the oysters were drinking at the 23rd hour and *B. typhosus* was recovered from the shell. This experiment shows no apparent immediate decrease in the number of *B. typhosus*. This apparent discrepancy is probably due to irregularity in contamination and to the accidental selection of oysters of low contamination at the beginning of the experiment.

TABLE 2.

EFFECT OF CHLORINE ON THE NUMBERS OF B. TYPHOSUS ON THE SHELLS OF CONTAMINATED OYSTERS

Tank	Time of Treatment	Temp.	Chlorine p.p.m.	B. typhosus per shell
A	0	19° C.	34.9	18
	1 hr.	14° C.	22.1	80
	3 hrs.	7.5° C.	15.6	70
	5 hrs.	5° C.	14.4	85
	24 hrs.	3° C.	8.1	10
	28 hrs.	5° C.	3.5	neg.
	48 hrs.	6° C.	2.1[1]	neg.
B	0	19° C.	34.9	18
	1 hr.	20° C.	16.5	100
	3 hrs.	20° C.	7.9	310
	5 hrs.[3]	21° C.	4.6	55
	24 hrs.	18° C.	1.1[2]	40
	26 hrs.	......	....	neg.

[1] A control tank without oysters contained 20 p.p.m. of chlorine.

[2] A control tank without oysters contained 7.9 p.p.m. of chlorine.

[3] Drinking began some time after the 5th hour.

NOTE.—B. typhosus could not be isolated from the chlorinated water after introduction of the contaminated oysters.

In experiment 4 (Table 3) a heavier contamination occurred than was expected. The concentration of chlorine employed was 15 parts per million. The results were similar to those obtained in the previous experiments, a marked initial decrease in the numbers of *B. typhosus* at the 1-hour test and very little apparent reduction in the tests subsequent to that. It should be noted that the contamination was so heavy that even the supernatant sea water was not rendered free of *B. typhosus*.

TABLE 3.

EFFECT OF CHLORINE ON THE NUMBERS OF B. TYPHOSUS ON THE SHELLS OF HEAVILY CONTAMINATED OYSTERS

Tank	Time of Treatment	Temp.	Chlorine p.p.m.	B. typhosus per shell
A	0	16° C.	15.8	143,500
	1 hr.	12° C.	6.9	5,390[2]
	3 hrs.	7° C.	2.4	1,450
	5½ hrs.[1]	5° C.	1.4	3,020
	24 hrs.	......	....	820
	27 hrs.[1]	4° C.	0.4	2,890
B	0	16° C.	15.8	143,500
	1 hr.	16° C.	6.0	4,150[2]
	3 hrs.	16° C.	0.7	1,250
	5½ hrs.	17° C.	0.3	1,440

[1] B. typhosus isolated from the tank water.
[2] Reduction partly due to rinse effect, see later experiments.

In experiment 5 (Table 4) the concentration of chlorine approximates that of the previous experiment (14 parts per million), but the contamination was very much lighter. *B. typhosus* was present on the shells at the end of an hour, and on the shells, in the liquor and bodies at the end of 6 hours. The oysters drank after the 1st hour.

TABLE 4.

EFFECT OF CHLORINE ON THE NUMBERS OF B. TYPHOSUS ON THE SHELLS OF CONTAMINATED OYSTERS

Time of Treatment	Temp.	Chlorine p.p.m.	B. typhosus per Shell
0	16° C.	14.5	395
1 hr.[1]	14° C.	3.9	94
3 hrs.	12° C.	0.9	65
5 hrs.	10° C.	0.3	54

[1] Oysters drinking between 1st and 3rd hour.

These experiments were disappointing and in themselves raised a grave question as to the efficacy of any chlorine treatment, as *B. typhosus* was isolated from the shells with only one exception before the oysters started to drink even though the initial chlorine content was as high as 30 parts per million in 2 of the experiments, that is, the degree of exposure was many times greater than that recommended by Wells.

Experiment 6 (not given in tabular form) really belongs to the above series. Although originally planned to determine the effect of successive chlorinations with intermediate drinking periods it was not continued. The oysters showed a minimum contamination, so slight, that even the rinse effect of sea water apparently was sufficient to clear the shells. In spite of this, *B. typhosus* was obtained from the bodies after at least 3 to 21 hours' drinking.

Experiment 7 (Table 5) was carried out with a slightly heavier contamination. Three exposures to chlorine were made, showing 8 parts per million, 24 hours later 12 parts, and again after 24 hours 12 parts per million. This experiment from the standpoint of the period of drinking as advised by Wells can only be considered as a double chlorination. However, typhoid bacilli could be isolated from the shells at every test up to 4 hours after the last chlorination. The liquors gave negative results 24 hours after the second chlorination but the bodies were still positive. This indicates that the flushing effect of the drinking may not result in the elimination of even small numbers of typhoid bacilli.

TABLE 5.

EFFECT OF SUCCESSIVE CHLORINATIONS ON THE NUMBERS OF B. TYPHOSUS ON AND IN OYSTERS

No.	Treatments Time of	Temp.	Chlorine p.p.m.	B. typhosus, Number per Shell	cc. of Liquor	cc. of Body Emulsion
1..........	0	16° C.	8.7	130	1.6	0.6
	1 hr.[1]	14° C.	1.2	35[3]	..	...
	3 hrs.	13° C.	0.6	11	...	...
	6 hrs.	11° C.	0.2	20	0.12	0.25
	24 hrs.	8° C.	0	4.3	neg.	+< 0.06
2..........	0	19° C.	12.7	...	...	...
	6 hrs.[2]	20° C.	0.4	6.9	0.19	0.19
	24 hrs.	18° C	0	2.5	neg.	0.125
3..........	0	19° C.	12.0	...	...	...
	4 hrs.	19° C.	1.4	2.5	neg.	+< 0.06

[1] Drinking between 1st and 3rd hour.
[2] Drinking not observed, opportunity during 18 hours.
[3] Dipping in plain sea water reduced the count to 19 B. typhosus per shell.

Owing to this failure of two exposures to moderate amounts of chlorine, experiment 8 (Table 6) was carried out with larger amounts. From 22 to 25 parts per million were used and a total of 4 exposures was given. The oysters were lightly contaminated. Typhoid bacilli were found on the shells up to 4 hours after the last (4th) chlorination but not 24 hours after

TABLE 6

EFFECT OF SUCCESSIVE CHLORINATIONS ON THE NUMBERS OF B. TYPHOSUS ON AND IN OYSTERS

No.	Treatments Time	Temp.	Chlorine p.p.m.	B. typhosus, Number per Shell	cc. of Liquor	cc. of Body Emulsion
1.........	0	10° C.	22.6	197	37	2.8
	4 hrs.[1]	11° C.	4.6	0.6[4]	0.24	0.25
	24 hrs.	11° C.	0.2	4.4	0.31	+<0.06
2.........	0 hrs.	......	22.4	...	...	...
	4 hrs.[1]	11° C.	7.6	1.25	+<0.06	.06
	24 hrs.	12° C.	0	1.88	0.125	0.37
3..........	0	......	25.2	...	...	...
	4 hrs.	12° C.	8.0	3.75	1.13	0.44
	24 brs.[2]	12° C.	1.4	1.88	0.3	+<0.06
4..........	0	......	22.9	...	...	...
	4 hrs.	12° C.	15.9	0.6	+<0.06	+<0.06
	24 hrs.[3]	11° C.	0	neg.	0.06	+<0.06

[1] Oysters drinking some time after 4th hour.
[2] Only a very short drinking period before rechlorination
[3] Probably had drunk for only short period.
[4] Dipping in plain sea water reduced the count to 30 B. typhosus per shell.

the 4th chlorination. The rate of fall of the chlorine during the first two treatments indicates that drinking was possible for at least a considerable part of the night. The only actual observation was that they were drinking in the morning. After the 3rd treatment the drinking opportunity was very short and the retention of chlorine after the 4th prevented drinking. This lessening of the rate of loss of chlorine after each treatment is noteworthy in other experiments in this series and is undoubtedly due to the gradual lessening of the organic matter through the rinse effect of the changes of sea water.

Apart from the shortness of the drinking period after the 3rd chlorination and its absence after the 4th, the experiment emphasizes again the weakness of chlorination as a means of freeing the exterior of the shell of the oyster of *B. typhosus*, in spite of amounts of chlorine and periods of action many times greater than recommended by Wells. With the slightly heavier pollution both the liquor and the body emulsion yielded *B. typhosus* to the end of the experiment. As in the previous experiment a few bacilli remained in spite of the drinking.

Experiment 9 (Table 7) was planned as a final test of all the factors. It was especially desired that by actual observation there would occur a drinking period after each chlorination in excess of 12 hours. It will be noted that the exigencies of the experiment resulted in much longer times in some instances. The almost constant temperatures were maintained by a continually flowing jacket of tap-water from the street main. This left no question as to dropping temperature on cold nights with inhibition of

TABLE 7

EFFECT OF SIX SUCCESSIVE CHLORINATIONS ON THE NUMBERS OF B. TYPHOSUS ON AND IN OYSTERS

Treatments		Chlorine	Drinking	B. typhosus numbers of per (with number of positive oysters in parentheses)		
No.	Time	p.p.m.	Observed	Shells	cc. of Liquor	cc. of Body Emulsion
1..........	0	10.9	...	248(9)	1.5(8)	+<0.04(4)
	4¼ hrs.	3.	No	...	...	...
	6 hrs.	1.4	Yes	...	...	...
	23 hrs.	0.2	Yes	2(9)	0.3(7)	+<0.04(6)
2..........	0	10.8	...	...	...	...
	3¾ hrs.	3.5	No	...	...	...
	5¼ hrs.	2.3	Yes	...	...	...
	6¼ hrs.	1.9	Yes	...	...	...
	23 hrs.	0.7	Yes	+<0.08(8)	0.3(8)	+<0.04(8)
3..........	0	10.9	...	...	...	...
	3¾ hrs.	5.5	No	...	...	...
	4 hrs.	...	Yes	...	...	...
	5¼ hrs.	...	Yes	...	...	...
	6¼ hrs.	4.1	Yes	...	...	...
	23 hrs.	2.8	Yes	...	...	...
	46 hrs.	1.8	Yes	1.3(9)	0.09(8)	+<0.04(4)
4..........	0	10.8	...	...	...	...
	6½ hrs.	3.9	No	...	...	...
	24 hrs.	0.7	Yes	...	...	...
	30 hrs.	0.5	Yes	...	...	...
	48 hrs.	Trace	Yes	+<0.08(6)	+<0.04(3)	+<0.04(1)
5..........	0	10.7	...	...	...	...
	5½ hrs.	2.7	No	...	..	...
	23 hrs.	0.3	Yes	...	...	...
	28 hrs.	0.2	Yes	...	...	...
	72 hrs.	0	Yes	4.7(7)	0.6(6)	+<0.04(6)
6..........	0	9.7	...	...	...	...
	4 hrs.	1.6	No	...	...	...
	6½ hrs.	0.9	Yes	...	...	...
	24 hrs.	0.3	Yes	1.3(5)	0.2(5)	+<0.04(8)

NOTE.—Dipping in plain sea water reduced the count to 57 B. typhosus per shell.

drinking. The experiment serves as a sort of *reductio ad absurdum* in that *B. typhosus* was isolated from the shells, from the liquor and from the bodies after a number of chlorinations and a period of treatment in excess of anything expedient in the trade.

A study of this table with special reference to the number and lengths of the drinking periods gives a discouraging view of the efficiency of the cleansing action of drinking, assuming that recontamination of the interior from the shells was not the factor. Whatever interpretation one may make, the practical fact remains that in this as in the previous experiments, a few typhoid bacilli persist inside of the oyster as well as upon the shells.

Conclusions

The chlorination treatment of contaminated oysters will result in a marked diminution in the number of *B. typhosus*, but even 6 successive treatments may not rid the oysters of the contaminating pathogens. The process cannot be recommended therefore in any sense as a reliable means of "sterilizing" contaminated oysters and thus rendering them safe for consumption. The results indicate that safe oysters can only be produced by growing them in waters uncontaminated by human feces.

A GRAM-POSITIVE ANAEROBIC BACILLUS—PRESUMABLY THE CAUSE OF A FATAL FOOD INFECTION.

Lucy Mishulow and M. Edw. Marten.*

A gram-positive anaerobic bacillus was isolated from the liver and spleen of a patient who died within 24 hours after eating in a restaurant. The patient complained of thirst soon after eating, then vomited. Shortly after that he developed psychosis and was sent to a hospital where he died within 24 hours after admission.

The autopsy showed as follows:

Some petechial hemorrhages of the skin of the body particularly on the left side of the abdomen.

The lungs showed intense congestion and edema with very marked petechial hemorrhages along the pleura.

There were intense hemorrhages retroperitoneally throughout the abdominal cavity particularly in the mesentery.

The bowels showed considerable hemorrhages.

The organism isolated from this patient is gram-positive in young cultures; but after 2 to 3 days incubation many gram-negative forms are found. It is a spore-bearing, capsulated and moderately motile bacillus, which grows only under anaerobic conditions. The bacilli in young cultures are about the size of the vibrion septique are fairly regular but in tissues and also in old cultures there are long filaments present.

It grows rapidly in nutrient media but only in the presence of a fermentable sugar.

Semisolid glucose-agar shake-cultures are almost entirely disrupted by the large amount of gas formed in 24 hours at 37°C. leaving a flocculent sediment in the bottom of the tube.

Glucose-broth is heavily clouded and very frothy after 24 hours incubation at 37° C. But on further incubation, the broth clears leaving a very heavy flocculent precipitate.

Spores are readily formed in the above medium; after 24 hours incubation the sediment contains numerous spores. They are oval and mostly central, although there are some subterminal spores. Some of them are larger in diameter than the bacilli thus giving them a spindle, or chlostridium appearance.

The colonies on glucose-agar are small, gray, and at first appear like a fine precipitate. On longer incubation they increase in size, but do not become very large even after two weeks incubation.

On blood-agar the growth is very delicate. The colonies are small, flat, transparent and non-hemolytic.

Litmus milk is acidified and coagulated after 2 to 3 days incubation at 37°C. with the production of gas which gives the clot a worm-eaten appearance.

Brain-medium is not blackened, and cooked-meat medium is not digested.

It does not grow in peptone-water even in the presence of a fermentable sugar.

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It ferments a large number of sugars. Table I gives the results of the fermentation reactions in extract agar, Ph 7.8 to which 1% of the test sugar was added. The inoculations were made from the sediment of a 24 hour glucose broth culture, a loopful of which was stabbed into the depth of the tube. In all the sugars that were fermented the reaction was complete after 24 hours incubation at 37°C. except in xylose, inulin, and glycerin where the reaction was not complete until 48 hours incubation. Where the reaction was negative the tubes were incubated for 10 days to rule out slow fermentation.

TABLE 1

FERMENTATION REACTIONS IN EXTRACT-AGAR—1% OF TEST SUGARS —ANDRADE INDICATOR

	Glucose	Levulose	Galactose	Lactose	Saccharose	Maltose	Raffinose	Dextrin	Salicin	Arabinose	Xylose	Inulin	Glycerin	Mannitol	Dulcital	Sorbitol
Acid...	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Gas....	++	++	++	++	++	+	++	++	++	++	+	+	+	−	−	−

Pathogenicity

This organism is not pathogenic for guinea pigs or rabbits. But a rabbit that was inoculated intravenously with a glucose-broth culture, killed 15 minutes after the injection and incubated at 37°C. overnight, showed characteristic post-mortem changes. There was bleeding from the nose and both ears were bright pink due to dilation of the superficial blood vessels. The subcutaneous tissues were intensely injected over the chest and thighs also the intestines showed areas of injection.

The bacilli were isolated from the blood, lungs, liver and spleen of the rabbit.

The organism is moderately pathogenic for mice. Table II gives the results on a series of mice that were injected intraperitoneally and subcutaneously and also were fed on the culture.

The results show that the culture is more pathogenic when inoculated subcutaneously than introperitoneally also that ½ to 1 c.c. of the culture when fed to mice will kill them in 2-4 days producing lesions that are similar somewhat to those found in the patient at autopsy.

There was little difference in the virulence between a 1 day and 14 day old culture thus indicating that there is apparently no extracellular toxin produced in culture.

This organism differs from any of the known anaerobes that are pathogenic when ingested, both in its biochemical and cultural characteristics, and also in the lesions that it produces in mice. Stoddard[1] described an organism very similar in morphology and biochemical characteristics which he isolated from a gas gangrene wound. He also found it non-pathogenic for guinea pigs and rabbits but he did not test it on mice and appeared to be doubtful as to its pathogenicity.

Unfortunately the food that the patient had eaten was not available for examination and therefore it could not be definitely proven that it was the cause of the infection. But judging from the results of feeding mice on the culture it seems possible that the ingestion of a large number of the bacilli in food might be the cause of a fatal infection.

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[1] Stoddard. J. L.—Bacillus multifermentans tenalbus. Lancet I:12. Jan. 4, 1919.

TABLE 2.

PATHOGENICITY FOR MICE

Mouse	Culture	Age of Culture	Method of Inoculation	Amount Inoc.	Result	Pathological Findings	Remarks
1.	Cooked Meat	24 hrs.	Introp.	½ c.c	Died in 4 days	Intestinal Injection	
2.	" "	7 days	"	1 c.c.	Died in 8 days		
3.	" "	7 days	"	½ c.c.	7 days	Hemorrhage from tail	
4.	Glucose broth	24 hrs.	"	1 c.c.	5 days	" " " Subcutaneous & Peritoneal injection	
5.	" "	24 hrs.	"	½ c.c.	Sick recovered		
6.	" "	24 hrs.	Subcut.	0.2 c.c.	Died in 6 days		
7.	" "	48 hrs.	"	1 c.c.	Died in less than 24 hrs.	Subcutaneous and intestinal inj.	
8.	" "	14 days	"	1 c.c.	Died in less than 24 hrs.		
9.	" "	14 days	"	½ c.c.	Died in less than 48 hrs.		
10.	" "	14 days	"	0.2 c.c.	Died in less than 72 hrs.	Hemorrhage from tail	
11.	" "	24 hrs.	Fed (Pipette)	0.2 c.c.	Remained well		
12.	" "	24 hrs.	Fed on Bread	1 c.c.	3 to 4 days	Paws, legs and tail bright red, also subcut. inj. over the chest	Bacillus isolated from liver and spleen
13.	" "	48 hrs.	"	1 c.c.	3 to 4 days	Paws bright red	Bacillus isolated from liver and spleen
14.	" "	48 hrs.	"	½ c.c.	48 hrs.		Bacillus isolated from liver spleen and intestine

THE DANGER OF DECOLORIZING VACCINE VIRUS

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Charles R. Tyler and Charles Krumwiede

In 1918 one of us in collaboration with Fielder and Watson reported observations on the use of brilliant green in vaccine virus. It was shown that the bactericidal action of this dye in concentration of 1–10,000 was a very potent means of obtaining a relatively rapid reduction of the bacterial content of vaccine virus or even obtaining practical bacterial sterility. This dye in this concentration was found to have no measurable effect on the virus itself.

These facts had been applied to some extent in the manufacture of our vaccine virus but the use of the dye had not been made a routine procedure because of the hesitancy of physicians to use a virus having a green color.

Due to the recent great demands for vaccine virus the expediency of the use of the dye led to its greater use and today we are employing it as a routine.

Coplans in 1922 published observations verifying our findings. He also noted that the green color was a drawback from the practical standpoint and investigated means of ridding the finished vaccine of the color. He recommended the use of sodium hydrosulphite as a decolorizing agent and stated that virus so treated remained potent during a period of fifteen months' storage at a temperature of 45° to 55° F. He stated, however, that some care is needed in the employment of this decolorizing agent, as an excessive amount of sodium hydrosulphite is capable of acting as a vaccinicide.

In extending our use of brilliant green we naturally had complaints about the color, and the recommendation of Coplans seemed worthy of observation and possibly of adoption.

Three lots of vaccine were decolorized by adding sodium hydrosulphite in a concentration of 0.25 gram per 100 c.c.; a quantity considered safe by Coplans. Clinical tests on children as well as rabbits were made over a period of three months; the vaccine being stored at 4° F. The continuously satisfactory "takes" seemed to warrant our marketing these viruses. They were accordingly filled in capillary tubes and vials and distributed.

Shortly we were in receipt of many complaints of vaccination failures. Because of the unfortunate failure of many physicians to keep a record of the lot or preparation number of the vaccine virus they use, there was a considerable delay before we became cognizant of what was wrong. It developed, however, that the decolorized viruses were at fault. This seemed inexplicable in the light of the fact that we were still obtaining 100 per cent "takes" in children and good takes on rabbits with the viruses diluted to 1–1,000.

There was this difference, however: the virus we were employing come directly from cold storage whereas the marketed virus had a delivery time of 24 to 48 hours during the warm weather of June. It seemed therefore that the virus must be defective in its resistance to higher temperatures. The available decolorized lots 361 and 363 were placed in the incubator at 37° C. for 24 hours and longer and then retested. As a control two other lots of about the same age and strength were simultaneously incubated. Reference to the table will show that the viruses containing the hydrosulphite became

RESULTS OF RABBIT VACCINATIONS WITH VACCINE VIRUS BEFORE AND AFTER INCUBATION AT 37° C.

Number and Character of Virus	Relative Potency—Rabbit Test Not incubated	Incubated 24 hours	 48 hours	 72 hours
361—Brilliant green and hydrosulphite	1-1000+++	1-10—	1-10—	1-10—
363—Brilliant green and hydrosulphite	1-1000+++	1-10—	1-10—	1-10—
366—Brilliant green	1-1000+++	1-1000+++	1-100+	1-10—
375—Brilliant green	1-1000+++	1-1000+++	1-10+	1-10—
388—Glycerol and Carbolic only	1-1000++++	1-1000++±	1-1000++±	1-1000++*
388—Brilliant green	1-1000++++	1-1000++±	1-1000++±	1-1000++±
393—Glycerol and Carbolic	1-1000++++	1-1000++++	1-1000++++	1-1000++++
393—Hydrosulphite 0.25 gm. per 100 cc.	1-1000++++	1-1000++++	1-10+±	1-10+
393—Hydrosulphite 0.025 gm. per 100 cc.	1-1000++++	1-1000++++	1-100++	1-10++
393—Brilliant green	1-1000++++	1-1000++++	1-1000+++	1-1000++
393—Brilliant green and hydrosulphite	1-1000++++	1-1000++	1-10—	1-10—

* After 96 hours the glycerol carbolic virus failed to take at a 1-100 dilution whereas the brilliant green virus still gave a moderate take at 1-1000.

Tests were made by cutaneous insertion of virus in dilutions of 1-10, 1-100 and 1-1000. Where takes occur they are recorded in the table in terms of the highest dilution giving a take and the degree of reaction with this dilution.

practically impotent within 24 hours, whereas the viruses containing brilliant green deteriorated to the same extent in 72 hours.

The pulp from two calves was collected and ground in glycerol-carbolic. These viruses were utilized for comparative experiments. The results are given in the table. In comparing the results it must be kept in mind that some irregularities will necessarily develop due to the varying susceptibility of different rabbits. This probably explains the apparent contradiction that with virus 388 the brilliant green virus did not deteriorate as rapidly as the glycerol-carbolic virus, whereas with virus 393 the opposite result was obtained. Continued observations have shown definitely that brilliant green in the concentration employed has no appreciable vaccinicidal action.

The experiments with virus 393 show very difinitely the vaccinicidal activity of sodium hydrosulphite. This virus had a much greater initial potency than viruses 361 and 363. For this reason naturally complete loss of potency did not occur so rapidly.

The last observation would seem to indicate that the combination of brilliant green and sodium hydrosulphite was more markedly vaccinicidal than was the hydrosulphite alone. As noted above, rabbits vary in their susceptibility and conclusion cannot be drawn from the one observation given.

SUMMARY

The addition of sodium hydrosulphite to brilliant green treated vaccine virus as a decolorizing agent is a dangerous procedure. If the virus so treated is kept at low temperature no deterioration may be noted. At higher temperatures, especially as they approach 37° C., the sodium hydrosulphite (or its cleavage products) shows a distinct vaccinicidal activity and tends to quickly destroy the potency of the virus. Its addition to vaccine virus which is to be marketed is therefore to be condemned.

For bibliography see the original article.

VACCINIA

CAROLINE R. GURLEY

Fifteen filtration experiments with vaccine virus are reported upon, together with control and sub-experiments.

TECHNIC

The samples of vaccine used were from the calf; they represented preparations used for human inoculation, after they had passed the prescribed bacteriological tests and had been recently tested for full activity. Each preparation consisted in a mixture of 0.4 gram of pulp with 4.6 grams of the following diluent: Glycerin 50 cc., water 49 cc., phenol 1 cc. This mixture was pressed through a brass sieve (40-to-the-inch mesh) and then through a sieve of a hundred to the inch mesh. For filtration the vaccines were diluted in most cases with physiological saline solution, usually after reducing the finely divided skin to a state of still finer division by grinding with sand. The degree of dilution employed is stated in terms of dilution of the pulp,—not in those of dilution of the vaccine as given above. The dilutions ranged from 1:12 to 1:50 or 1:60, the usual dilution being about 1:25. When the filtrate was to be concentrated by evaporation in vacuo, 0.5 saline solution (instead of 0.85) was employed.

Berkefeld filters (Grade V) were employed. Every suspension of virus was inoculated, before filtering, with an emulsion of B. prodigiosus (occasionally with staphylococcus instead) to test the integrity of the filter; cultures of the filtrate (1 cc. on each slant) were made to determine presence or absence of the organism.

Clogging of Berkefeld.—Great difficulty arises from clogging of the filter by the finely divided skin present in the virus mixture, though this trouble is much less when the virus is not sandground. The following ways of clearing the material were tried (a) long centrifuging; (b) filtration through paper, paper pulp, shredded asbestos, alundum cups, and sand,—the last used by itself or mixed with a small amount of animal charcoal. The first four methods of filtering failed because of quick clogging and sand did not remove the fine particles. Brushing the surface of the candle with a stiff brush is not applicable during positive pressure work, though a help during suction work. During long centrifuging (1½ hours at 2040 revolutions) the organisms appeared to be carried down in large proportion by the sedimenting skin-particles. The addition to the cloudy fluid, of barium sulphate and also of starch was tried with two portions of virus, in comparison with a third portion which received nothing. The tube containing the sulphate cleared more quickly and thoroughly on centrifuging, than the other two, between which there was little difference. The supernatant fluid containing the virus from all three was passed through a Berkefeld V with a positive pressure of somewhat less than 50 pounds, (the desired pressure aimed at), and then inoculated intradermally into rabbits with the following results:—

Virus freed from skin particles as follows	Rabbit	Result
By addition of $BaSO_4$ and centrifuging	A B	2 Small vesicles Negative
By addition of starch and centrifuging	A B	Definite "take" Negative
By centrifuging only	A B	Definite "take" Negative

The fact in this experiment that of two rabbits which received exactly the same material, one was positive, although the take was slight and the other not, probably indicated both a difference in susceptibility of the rabbits and that few organisms passed the filter.

Attempt to clarify by electric current.—Separation of organisms from skin particles was tried by this means. Saline emulsion cannot be used because of course free chlorine is evolved. Hence glycerin and water mixtures were tried,—first, 50 per cent., then 10 per cent., then 5 per cent. With a current of seven milliamperes, passed through 50 per cent. glycerin emulsion, a collection of the organisms (*also of the colloidal skin particles*) took place around the cathode; or possibly there was a killing of the organisms around the positive pole. Three areas on a rabbit inoculated with negative pole fluid, at 10, 20 and 35 minutes of passage of the current respectively, showed good confluent takes, while the 4th area (positive pole fluid withdrawn at 40 minutes) showed *one* good vesicle, and no other sign. At termination of the experiment, the fluid through which the current had passed was distinctly acid to litmus.

Use of electrolytic substances.—Realizing that the clarification by the current was due to the generation in the fluid of minute quantities of electrolytes, an attempt was next made to clarify by introducing electrolytes directly. Cerium chloride and copper sulphate were used, although little hope of success was entertained with the latter. It produced perfect sedimentation after two minutes centrifuging with a water-clear supernatant fluid but inoculation with this un-Berkefelded fluid did not yield a result good enough to justify its further use. Cerium chloride (¼ of a cc. of .025 per cent. solution per cc. of the skin emulsion) left the emulsion still cloudy after three minutes centrifuging at 2040 revolutions, but it proved clear enough to pass the Berkefeld with fifty pounds pressure. Two filters were used, part going through each. These filtrates did not yield a take. UN-Berkefelded control material inoculated intradermally, gave many good vesicles. While the negative result with the Berkefeld V filtrate was distinctly disappointing, this method of clearing should hardly be abandoned without further trial, as adsorption by the filters may have caused the negative result, which might have been different had all the fluid been passed through one filter.

Another attempt at preparing the fluid for the Berkefeld consisted in filtration through alundum cups. This was done primarily to see whether the virus would pass through these relatively coarse filters, as we had then met with no positive results with Berkefeld V. Three experiments were done, all by suction. In one case the rabbit inoculated with the alundum-cup filtrate gave a very fair take, in the second case the take was poor but definite; in the third, the filtrate was passed through Berkefeld V and, when afterward inoculated, gave no result. Each time, B. prodigiosus passed through the cup together with the virus. In fact, the third experiment was done to test the relative coarseness of cups of different grades (there are three) by means of an emulsion of B. prodigiosus. The grading done by the firm manufacturing these cups proved untrustworthy from the filtration standpoint. Thus, of 2 cups of the same grade, one held back a considerably larger percentage of bacilli than the other. Cups graded alike differ in weight, and in apparent density to the naked eye. Poured plates from filtrate through cup RA 84 supposed to be denser than RA 360, representing dilutions of 1-10, 1-1,000, 1-100,000 all showed prodigiosus; plates from RA 360 filtrate showed growth except in 1-100,000. RA 98 gave growth in all 3 dilutions; it was supposed to be the coarsest. When the filtrate from both cups (Ra 84 and RA 360) was pooled and a rabbit used, no take resulted.

Concentration of filtrate in Vacuo.—This can be done over sulphuric acid. Phosphorus pentoxide was used in 2 cases but possesses no advantage over sulphuric acid. A mixture of yellow wax and paraffin was used for sealing the dessicator. A fair degree of vacuum was easy to obtain, one corresponding to 745 mm. of mercury, but leakage may occur. The best make of dessicator must be employed, preferably one in which the sulphuric acid can be placed above the fluid to be dried (Hempel's style). With a good vacuum a great concentration or even evaporation to dryness takes place over night, unless a large quantity of filtrate is used; about 14 cc. in a petri dish became dry.

Analysis of Experiments

Fifteen Berkefeld V filtrates were inoculated on to the skin of 28 rabbits; four definitely positive results (14%), one probably positive, one somewhat suspicious and two very questionable "takes" were obtained.

Suction, and positive pressure up to fifty pounds were tried. Suction was accomplished by a Censo-Nelson vacuum pump.

Filtrates giving positive results.—Of the four filtrates yielding certain takes, two represented virus from one calf, and the other two represented virus from each of two other calves.

Character of the "take" resulting from inoculation with filtrate.—Only one take was obtained by rubbing filtrates into the skin. Other successful inoculations were intradermal, which method allows of 5 or 6 cc. being inoculated upon one rabbit. We, however, inoculated from 1½ to 3 cc. of each filtrate intradermally per rabbit, sometimes using other areas on the same animal to test rubbing-in of the same fluid (1 cc).

The reactions yielded by filtrates differed somewhat in different rabbits, and differed considerably from those of a good "take" obtained with unfiltered virus. The filtrate reactions consisted of inflamed areas raised little or not at all above the surface, but usually fairly well circumscribed. When excised, these areas were found to be hard nodules. In one or two cases when the areas were not circumscribed, it was doubted whether the few inflamed spots did constitute a "take". Both in these cases and in ones thought to be positive, the inflamed areas developed at the proper time as indicated by the incubation. To prove that these constituted a "take", they were excised, ground with sand, a saline suspension made, and—after clearing sufficiently by centrifuging—inoculated into one or more rabbits. The resulting nodules were more numerous, larger and more definite in these rabbits representing the second generation, and developed at the proper time (third or fourth day); in the first generation the nodules were some to many times fewer than were the spots injected. In one second generation rabbit the nodules were extremely large and much inflamd; in smears stained by Gram no bacteria were found.

The atypical appearance of the "takes" with filtered virus was believed to be due to the high dilution resulting from adsorption by the filter. To test this, dilutions were made, running from 1:300 to 1:10,000 of the virus as regularly prepared, that is, .4 gram of pulp to 4.6 cc. of 50 per cent. glycerin solution containing 1 per cent. phenol, or 1:3,450 to 1:115,000 of the pulp itself. These dilutions were inoculated both intradermally and by rubbing in.

The dilutions of 1:1,000; 1:3,000; 1:5,000 and 1:10,000 showed no definite takes on the rubbed-in areas, but showed the discrete inflamed nodules, not projecting, which resembled takes from filtrates, although the latter were less regularly circumscribed.

Positive Filtrate No. 1 (Calf virus 237; Jan. 20, 1921).—Dilution of the virus, by which is always meant total dilution of the pulp, was 1:24 sand-ground 5 minutes; cleared for filtration by centrifuging; Berkefelded with 25 pounds pressure; inoculated on to one rabbit, both intradermally (1½ cc.) and by rubbing-in very thoroughly with the finger (1 cc.—all that skin-area would absorb). As both of these areas were on one side of the animal, it cannot be said with certainty that the take was limited to the rubbed-in area, but—contrary to expectation—in this case the preponderant take was there. On 4th, 5th and 6th days, areas regarded as distinctly suspicious were present; on the 6th day these nodules were excised for reinoculation, which gave an unquestionable take. After most of the diluted virus had gone through under 50 pounds pressure, the small remainder was diluted so that it represented a dilution of 1:48, and was passed through the same filter by suction, the surface being brushed constantly with a rather stiff brush to prevent clogging. The rabbit inoculated with this second filtrate gave a questionable take. Reinoculation was not done.

Positive Filtrate No. 2 (Calf virus 200; Jan. 25th, 1921).—Dilution of virus 1:26; sand-ground five minutes; cleared for filtration by centrifuging; divided into two portions, A and B, both passing through the same filter; A under twenty-five pounds and then B under fifty pounds pressure. One rabbit was inoculated with each filtered portion. The twenty-five pound-pressure rabbit remained perfectly negative until the 11th day when there was one small suspicious area, but two days later this area no longer appeared suspicious. The fifty pound rabbit on the 4th day showed definite nodules on the intradermal area and was considered positive. These were excised and ground with sand as usual, for reinoculation, and the result was unquestionably positive. The rubbed-in area was negative.

Positive Filtrate No. 3 (Calf virus 196; March 29th, 1921). Dilution of the virus to a smaller degree than in the other experiments that proved positive; sand ground five minutes; cleared by centrifuging and Berkefelded with fifty pounds pressure; inoculated intradermally into two rabbits; each receiving 1½ cc. On the sixth day one rabbit showed a suspicious take, which on the seventh day was regarded as positive. Inoculation of excised areas into three other rabbits gave positive results. The other animal was negative, except that on the fourteenth day very questionable small areas were noticed.

Positive Filtrate No. 4 (Calf virus 196, April 6th, 1921). Dilution of virus 1 to 48; not sand-ground; cleared for filtration by centrifuging. Berkefelded under about forty pounds pressure; inoculated intradermally into two rabbits, each receiving 1½ cc. On the fifth day one rabbit was considered probably positive; on the seventh day certainly positive. Reinoculation gave an undoubted take. The companion rabbit remained negative. It was watched for fourteen days.

Two of the above four filtrations were done with fifty pounds positive pressure, one with somewhat under that (fifty pounds were aimed at, but the fluid passed through quickly before that amount was reached), and one with twenty-five pounds. Two of these filtrates represented a dilution of, practically, 1:25; one a dilution of 1:48; and the fourth, probably a much stronger concentration than any of the others. The dilution in this instance was unknown because the virus used was the sediment packed down by the centrifuging when dealing with a 1:48 dilution. This sediment (the supernatant fluid from which was separately Berkefelded for inoculating another rabbit with negative result) consisted of finely divided skin and sand-particles. It was washed with only 4 cc. of saline solution, re-centrifuged, and Berkefelded.

Filtrates giving questionable results.—The rabbits classified as probably positive (also that regarded as somewhat suspicious,—see below) received filtrates obtained by suction.

The probably positive filtrate represented a dilution of about 1:40; was sand-ground, centrifuged, Berkefelded and evaporated slightly in vacuo—about ⅕ to ¼ of its volume. The one rabbit inoculated received 1½ cc. intradermally (also a small amount rubbed in) on each side; 4th day negative; 6th day, few small but distinct vesicles on right side (excised); 7th day, three more very small nodules had appeared. The transmission experiment was not successful (except for 2 very small questionable nodules on one of the two rabbits) but this is more probably attributable to a defect in carrying it out (no sand-grinding was done) than to the nodules not having represented vaccinia ones.

The filtrate which gave a suspicious take represented a dilution of 1:33; was sand-ground, centrifuged, Berkefelded by suction, and thereafter centrifuged 1½ hours. The portion from the bottom of the tubes was inoculated in two rabbits. One was negative for seven days, on the eighth was somewhat suspicious; on the 10th negative. The companion rabbit was negative. The experiment included two other rabbits one of which gave a questionable take. This rabbit received the supernatant fluid, after the 1½ hours centrifuging, which was evaporated in vacuo, inadvertently *to dryness;* emulsified with water, and about ½ cc. injected intradermally. On the third day, and later, there was suspicion of a reaction.

The fourth animal received the filtrate uncentrifuged and unevaporated, and was negative.

A higher percentage of positive filtrates might have resulted except for such circumstances as these: The clearing necessary in order to prevent clogging, as, for example, filtration through paper pulp or long centrifuging may have robbed the virus of a considerable proportion of its strength. In other cases in which the clearing was imperfectly done, the filter soon clogged, causing, perhaps, the same negative result.

Evidently, unless a method of separating virus from accompanying skin is obtained, filtration to free the virus from other organisms is not practical, except for obtaining small amounts of material for culture experiments.

LABORATORY OBSERVATIONS ON THE VIRUS OF THE MALIGNANT DISEASE RECENTLY EPIZOOTIC AMONG POULTRY

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Charles Krumwiede, Herman Gerber and Dorothy J. Provost

When, in September, 1924, an epizootic of a malignant infectious disease devolped among the market poultry in New York City, Dr. Aaron Silkman, Chief Veterinarian of the Department,[1] requested the cooperation of the Bureau of Laboratories to investigate at least two aspects: first, the etiology of the disease; second, the possibility of harm to man. The following report gives the experimental data obtained.

The clinical and postmortem picture of the disease gave every indication that it was septicemic in character. The hemorrhagic lesions suggested the possibility of an infection by one of the members of the pestis group. Chickens were obtained and examined bacteriologically. The chickens used for this purpose were either killed when very ill or examined promptly after death, so as to eliminate postmortem invasion of the blood. Eighteen chickens were thus examined. Direct smears of the blood were negative. Cultures were made from the blood and liver on the various laboratory media, especially on blood-agar. In the majority of instances a very few colonies developed. A study of these indicated that some were most probably contaminations obtained in the process of autopsy and culture. The bacterial varieties more frequently encountered in the cultures were a Gram-positive coccus (4 chickens), a Gram-positive diphtheroid (7 chickens), and a small, slender, Gram-negative bacillus with a slight tendency to polar staining (5 chickens). The former two varieties, when found, were very few in number and were so presumably contaminations from the skin and feathers that they were held but not further studied, except that a chicken was injected with 1 cc of a broth culture of the coccus, without effect. The last variety, the Gram-negative bacillus, seemed the most likely infecting agent, as the colonies per agar plate ranged from 2 to 20. This variety caused hemolysis of the blood.

Broth cultures of this bacillus were injected subcutaneously or intraperitoneally into five normal chickens without effect. The maximum dose was 2 cc. These negative results indicated that none of the bacterial varieties was the causative agent of the disease and no further study of these cultures was made. Cultures of the intestinal contents had also yielded nothing distinctive.

The failure to demonstrate the existence of a bacterial infection of the blood in a disease so distinctly septicemic in character was seemingly conclusive and turned our attention naturally to the probability of a filtrable virus.

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[1] We are indebted to Dr. L. D. Krohn, who carried out the field investigations and supplied us with the infected and normal fowls for this work.

Filtration Experiments

Blood was collected, therefore, from chickens, killed during the late stages of the disease. This blood was defibrinated and after dilution with four volumes of broth was centrifuged to sediment the red cells and filtered through small Berkefeld filters. Filtration was accelerated by suction, using the filter-pump on the water-faucet. The negative pressure obtainable on the faucet used was 15 inches of mercury. The filtration results were controlled in all instances by cultural examinations. In the first filtrations the presence, before filtration, and the absence, after filtration, of the hemolytic bacillus above described, indicated that the filters were satisfactory. This was not considered a sufficiently rigid control. Subsequently each collection of the blood was very heavily contaminated with *B. prodigiosus* and from 1 to 2 cc. of the filtrate was tested and found sterile. This very rigid test excluded the possibility of leakages and also excluded the possibility of "faults" developing in a filter previously found to be efficient.

Seven such filtration experiments were carried out. In most of the experiments one chicken was fed and one was injected subcutaneously with 2 cc of the unfiltered material and one chicken was usually fed and one injected subcutaneously with 2 cc of the filtered material.

The following summarizes the results:

Blood virus unfiltered, fed to 6 chickens; 5 died, 1 immune.

Blood virus filtered, fed to 6 chickens (1 received 6 cc); 6 survived (1 found immune on subsequent test).

Blood virus unfiltered, injected into 6 chickens; 5 died, 1 immune.

Blood virus filtered, injected into 7 chickens; 6 died, 1 immune.

That we were actually dealing with a multiplying virus and not a filtrable toxic substance was corroborated by the passage of the disease from chicken to chicken after filtration, and by passages with intermediate controlled filtration of the blood. All the chickens in these and subsequent experiments, unless otherwise noted, were kept singly in cages to eliminate the possibility of contact infection. Likewise, all inoculated birds were quartered in a different building than that housing the unused supply. In nearly every instance where birds survived an inoculation, they were again injected with a large dose of virus to determine whether the failures were actually due to an immunity.

These experimental data leave no doubt that the epizootic disease in question was due to a filtrable virus, filtrable in the sense that it passed through a Berkefeld filter under the conditions of the experiments.

Loss of Virus During Filtration

The experiments also show that the virus will cause the disease when either fed or injected. A comparison of the results of feeding experiments, before and after filtration, indicate (see below) that there was a distinct loss of virus during filtration. No attempt was made to determine the mechanism of this loss, especially as regards parallel experiments with filters of smaller porosities. A comparison of the feeding and injections after filtration, in the light of further data given below, shows that feeding of the virus is successful only when more virus is present than is necessary to cause infection by subcutaneous injection.

Having definitely shown that the disease was due to a filtrable virus, the next point investigated was the virulence of the virus. Dilutions of the defibrinated blood obtained from dying chickens were tested in increasing dilutions. The subcutaneous injection of as little as 0.000,000,001 cc of blood was found sufficient to cause the disease. On feeding, 0.01 cc caused death, but 0.001 cc was followed by survival.

Not only has the virus extraordinary virulence, as shown by the above data, but it also has an equally high invasive capacity. Several experiments showed that the slightest scratch of the skin, followed by a mere application of the blood virus, was sufficient to cause the disease. As will be noted later, the oral discharges are infectious. A chicken dead of the disease was taken and his bill sharply tapped against the skin (bared by laying aside the feathers) of a normal chicken. This was sufficient to cause the disease. A drop of defibrinated blood, dropped into the eye, also caused the disease in two chickens.

The virulence of the virus for other species of fowl was also tested. These results are incomplete, as the actual minimum infecting dose was not reached because of insufficient experimental material. This was due largely to the fact that we were misled by the apparent natural immunity of these species and used too large doses in most instances.

The smallest doses, of those tested, which caused death on subcutaneous injection were:

Pigeon	0.00,000,001 cc.
Turkey	0.00,001 cc.
Ducks	0.5 cc died, 0.1 cc survived
Geese	0.1 cc (one goose survived; one goose died)
Guinea hen	0.0,000,001 cc.

The time of death in these species averaged close to 48 hours after inoculation. That the above deaths were due to the virus was controlled by subinoculation into chickens, with development of the typical disease.

The virulence of the virus for mammals was also tested. Mice injected intraperitoneally with 0.5 cc. of defibrinated blood virus survived. Guinea pigs receiving 1 cc of blood virus intraperitoneally also survived. Rabbits were injected with 1 cc. of virus intravenously without effect. Two monkeys *(Macacuc)* were each injected with 4 cc. of defibrinated blood subcutaneously and also fed with 3 cc. of the virus. They showed no ill effect. These observations indicate that mammals are naturally immune and that the virus is harmless to man.

Some observations on the excretion of the virus from infected chickens were also made. The oral mucous secretion contains the virus. This secretion, when mixed with broth, caused death on subcutaneous injection. The skin of a chicken was slightly abraded and a swab carrying the secretion was applied; this also caused the disease. It was previously noted that tapping the skin of a chicken with the beak of a dead chicken caused the disease. A swab contaminated with the oral mucous was gently applied to the mucous membrane of the mouth of a well chicken. This sufficed to cause the disease.

The feces apparently contain the virus but not in great quantity or regularity. Feces scraped from the cage bottom, on death of a bird, were emulsified in broth and injected into two birds, of which one survived and one died. The bird that survived was not an immune. The difficulty in obtaining feces from caged birds and the possibility of contamination of the cage bottom with oral secretion led us to attempt a better method. A bird was placed in a jar when evidently ill and the dropping collected immediately when observed. The injection of an emulsion of this material caused death. The deaths following the injection of feces were controlled by smear and culture examination to exclude bacterial infection and by subsequent injection of the blood of dead chickens into normal chickens, which caused the typical disease. The amount of virus was apparently not great as the birds injected with feces did not die till close to 72 hours.

NATURAL SPREAD OF DISEASE

Some of our observations are suggestive of the mode of transfer under natural conditions. These are the presence of the virus in the oral secretions in considerable amount; the presence of the virus, though apparently in less amount, in the feces; the production of the disease by contamination of the mucous membranes of the eye or mouth; the extraordinary ease of infection through slight abrasions of the skin. It would seem that oral infection, through contamination of food and drink, with mouth secretions or through the actual injection of the blood or organ virus obtained from dead birds, was a factor. Injuries of the skin by any means, with contamination of the injury, would also be a factor. We were surprised, however, that our attempts to cause contact infection failed. Two chickens, caged with an inoculated bird which died in the cage, did not contract the disease. This cage was bedded with hay. Four birds, similarly exposed in a cage without bedding, also failed to contract the disease. In both these experiments the contacts were left in the presumably contaminated cages for a week or longer. That they were not immune was shown by subsequent inoculation.

Dr. Krohn, in the preceding article,[1] describes the postmortem lesions in detail. The picture in our experiment birds was similar. The hemorrhagic lesions of the stomach and heart were present in practically every one.

The experiment birds developed a very acute form of disease. The great majority of the birds died in from twenty-four to forty-eight hours. With minute amounts of virus, death was delayed to 72 to 96 hours. No chicken which developed symptoms of illness survived. Illness was not observed, however, in many birds. With some the disease developed and death occurred out of laboratory hours. Frequently birds would be observed that appeared apparently well, only to be found dead several hours later. Where evident illness occurred, death usually ensued very quickly.

A considerable number of varieties of chickens as well as mongrels were used in the experiments. No differences in susceptibility were noted under the conditions of our experiments. It should be noted, however, that the majority of the injections were of considerable amounts of virus. We encountered very few immune birds. The great virulence of the virus under laboratory conditions raises an interesting question as to the mechanism of such an immunity.

There was a strong suggestion that the virus increased in virulence in the laboratory passage from chicken to chicken. This was indicated by the tendency to the shortening of the period of life in our later experiment birds. Over fifty virus passages have been made.

DISEASE PROBABLY FOWL PEST

The question naturally has been raised as to the nature of the disease. Dr. Krohn has discussed this from the standpoint of symptomatology and pathology and has drawn a close parallelism with fowl pest. Our laboratory observations on filtrability of the virus, its virulence, invasive capacity and excretion closely parallel those given by Ostertag. Our species pathogenicity experiments are, however, somewhat different in some details. A tentative conclusion is justified that the disease is fowl pest, though a final conclusion would have to rest on a study of the foreign and domestic viruses, under parallel conditions.

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[1] Journ. of The Amer. Vet. Med. Assn., 1925, Vol. LXVII, pp. 146-170.

Besides our preliminary note on the existence of a disease in chickens due to a filtrable virus, which appeared January 31, 1925, only one other report has appeared, as far as we know, on the etiology of the disease. Brunett offered a preliminary report on his work. His bacteriological examinations were negative. He found that the injection of "minute" amounts of blood caused the disease; likewise the feeding of chopped liver of a diseased bird. He concluded that the disease was due to a filtrable virus. This conclusion he based on two filtration experiments, the filter having been previously tested by ascertaining its ability to retain *Pasteurella avisepticа.* He carried the virus through nine generations but he does not state whether these included the filtered material.

SUMMARY

The recent malignant epizootic disease in poultry was due to a filtrable virus. This virus was highly virulent; 0,000,000,001 cc of blood virus, injected subcutaneously, or 0.01 cc of blood virus, given by mouth, caused death. The mere application of virus to slight abrasions of the skin sufficed to cause infection and death. The virus is present in the oral secretions and also in the feces in lesser quantity. Other species of fowl are susceptible to a varying degree. Mammals are immune. The observations recorded strongly indicate that the disease is the European fowl pest.

For bibliography see the original article.

A DROPPING BOTTLE AS AN AID IN MACROSCOPIC SLIDE AGGLUTINATION

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Charles Krumwiede

The macroscopic slide agglutination method has been more or less generally known for years. The value of the method, however, has not been generally appreciated, probably due to the fact that most of the serums employed for indentification purposes have not been sufficiently active to give an immediate or prompt agglutination even in low dilutions. Coca utilized the method in cholera examinations and, to our knowledge, he was the first to publish a description of the method. We have utilized it for some years as a routine in examinations of feces for members of the typhoid-paratyphoid dysentery types and also as an aid in the search for meningococcus carriers. As very potent sera become more generally available its use will undoubtedly spread to most laboratories.

There is one inconvenient feature in the method which we believe to have overcome by the device here reported. In placing on the slide the drops of diluted agglutinating serum and the saline or diluted normal serum for control it has been the custom to utilize the ordinary platinum loop. Where many colonies are to be tested this method is time-consuming and tedious.

The dropping bottle that we have devised to deliver the drops depends upon the use of a capillary delivery tube with a flat end and a rubber diaphragm to force the delivery of the drop.

The delivery tube is prepared from a piece of heavy walled tubing (thermometer type) having an outside diameter of about 4mm. and a bore of about 1mm. This is heated thoroughly in the flame and drawn out slowly. A piece is then cut as shown in the outline sketch (figure 1) and the larger

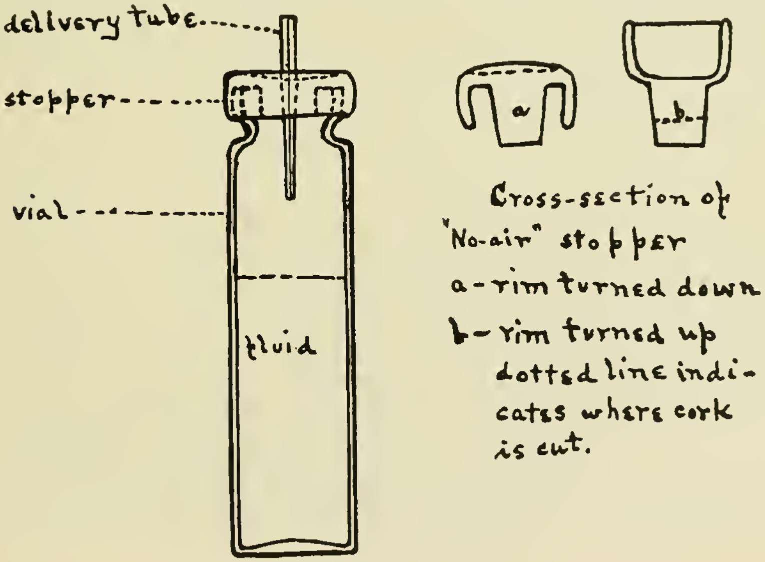

Fig. 1.
(Reprinted by courtesy of Journal of Immunology.)

end ground flat and smooth on a fine stone. The diameter of the bore is reduced to about the bore of 20 gauge hypodermic needle.

The diaphragm is made from a so-called "no-air" stopper, size No. 2½. The cork end is cut off leaving only sufficient to give stability to the delivery tube. The delivery tube is thrust through the cork.

The bottle employed is the regulation ½ ounce wide mouth homeopathic vial. The neck of the bottle must be wider than the cork portion of the stopper to allow up and down play otherwise there will be no diaphragm action.

The bottles are partly filled with the diluted serums or saline and the drops are placed on the slide by holding the bottle vertical and pressing till the appropriate sized drop is delivered. With very little practice many drops can be delivered in a few seconds. The appropriate pressure to apply soon requires no conscious attention.

The amount of air or air tension in the bottle needs readjustment from time to time. Air can be introduced by inverting the bottle and pulling on the delivery tube. If there is too much air pressure this can be equalized by holding the bottle upright and pressing down the diaphragm which empties the tube and results in equalization of the pressure.

There are other possible applications of this dropping bottle. It may be utilized to deliver drops of saline or other fluids used in the preparation of smears of cultures. In the classrooms it could be utilized to give out suspensions of microörganisms for smear examinations. Other similar uses will probably be found for it.

For bibliography see the original article.

A NEW METHOD OF ADDING CRESOL TO ANTITOXINS AND ANTISERUMS

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Charles Krumwiede and Edwin J. Banzhaf

As is well known, the addition of cresol to antitoxins and other antiserums results in the formation of a precipitate due to the combination of the cresol with the proteins. This precipitate is greatest when all the cresol needed to give the desired concentration is added rapidly. To avoid this more or less voluminous precipitate, various methods have been resorted to. The cresol may be added in a dilute form. This method has the disadvantage of diluting the product to which it is added. Another method is to add the undiluted cresol drop by drop to the product. In this method the serum is commonly placed in an open vessel and beaten with a rod or paddle as the cresol is added to obtain thorough mixing. Similarly, admixture is often accomplished by adding the cresol drop by drop to the large stock bottles, replacing the cork and shaking the container thoroughly after each addition. These methods are not above reproach, as they open the way to contamination. Different producers have devised various methods to limit the exposure to contamination during the addition of the preservative. It is evidently essential that the amount of precipitate formed should be limited as much as possible. In the first place, the precipitate binds up the preservative and thus lessens the amount available throughout the product. How much remains in the serum is not known. Furthermore, the precipitates increase the loss of the product in the process of filtration and add as well to the difficulty of this process.

These considerations raised the question whether we could not find a better method for the addition of cresols to antitoxins and serums. After several preliminary experiments, ether was tried as a solvent. It was found that equal parts of cresol and ether are miscible and make a permanently clear solution. On the addition of such a mixture to antitoxin or serum, the solution floats on the surface causing a slight haze at the point of contact. If shaken immediately after the addition either no precipitate or at most very little is formed. Even the rapid addition of all the solution needed to give a concentration of 0.4% to 0.5% of cresol, causes at most only a slight precipitation; the amount of which is in no way comparable to that resulting from the rapid or even drop by drop addition of the same amount of cresol alone.

With this success in eliminating precipitation when the whole volume of preservative was quickly added, several questions immediately arose: First, what would be the effect of the additional ether when the product is administered? Second, would the product, on aging, precipitate, or in other words would the cresol slowly but eventually cause as much precipitate as if it alone had been added at first? Third, would the loss of ether during filtration and other processes result in objectionable precipitate? Fourth, would the bactericidal action of cresol be decreased by the ether or not? If not, would the additional bactericidal action of the ether be of value? Fifth, would the ether affect the potency of the product?

No untoward clinical effects follow the use of serums containing ether in the concentration noted, even when administered intravenously or intraspinally.

As to the subsequent development of precipitation in antitoxins and serums to which ether-cresol has been added, we can at least say that it is no greater than had cresol been added alone.

The amount of ether lost during the processes of filtration, that is, filtration through paper pulp and through the Berkefeld filter, has not resulted in the formation of a precipitate during the stages of the process or as it finally comes from the Berkefeld filter. The filtered product is crystal clear.

In regard to the fourth question, namely, would the ether lessen the bactericidal action of the cresol or would it leave it unchanged, and if the action of the cresol were unchanged, would the bactericidal action of the ether make the mixture appreciably more active, the disinfectant tests show that the mixture is more efficacious than cresol alone.

On the whole, our experience and that of others who have tried the combined ether-cresol preservative, indicates that the increased bactericidal activity is of decided practical importance, especially in relation to those products which, because of their mode of preparation, cannot be kept sterile.

Experience has shown that the potency of serums and antitoxins is uninfluenced by the ether. Preparations of diphtheria antitoxin, antimeningococcus serum and of antipneumococcus serum have been retested as long as 7 months after addition of the ether cresol. The ether may be added to a toxin-antitoxin mixture without interfering with the balance as the ether is without effect on the more labile toxin element.

Certain theoretical considerations have arisen while collecting the purely practical facts outlined in the foregoing. These points may be mentioned. On mixing the ether and cresol, considerable heat is generated. We have not attempted to determine whether any new compound is formed. If the mixture consists of 25% of ether and 75% cresol, instead of half and half, the addition to serum results in a marked opalescence of the serum although marked precipitation may not occur. Ether tends to reduce the surface tension of the serum. This may possibly be a factor not only in inhibiting precipitation but also a factor in the apparent inhibition of reaction after the intravenous administration of antitoxin. We have not attempted to give a summary of the previous use of ether as a preservative nor of the use of ether in the prevention of anaphylactic shock in experimental animals. The former is generally well known. The latter would involve a discussion which we wish to avoid by simply presenting the practical results obtained without any attempt at explanation.

Summary

A mixture of equal parts of ether and cresol is presented as a new preservative for antitoxins and serums. This mixture is added in amounts necessary to give the required concentration of cresol. The addition of this mixture causes much less precipitate than does cresol alone. Subsequent precipitation is not necessarily limited by the ether, it is never greater than that in products containing cresol alone. The mixture of ether and cresol is more strongly antiseptic than cresol alone. In therapeutic application, the ether is not a disadvantage. In the case of intravenously injected antitoxin, the indication that the ether may under certain circumstances reduce the incidence of adverse reaction warrants further comparative work. Ether may be added to the toxin-antitoxin mixture without disturbing the balance of the mixture.

A COLORIMETRIC REACTION BETWEEN GOLD CHLORIDE AND TOXINS APPARENTLY INDICATIVE OF TOXIC STRENGTH

A Preliminary Report

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Lucy Mishulow and Charles Krumwiede

While we were working on the effects of different agents for detoxifying diphtheria toxin, a report appeared by Osman[1] stating that gold chloride would prevent the toxic reactions usually following the injection of bacteria. This led us to try this agent with diphtheria toxin. In testing the effect of the gold chloride in different concentrations, it was noticed that color reactions occurred. As we were at the same time enagaged in the investigation of the possibility of titrating toxin and antitoxin by physico-chemical means, the idea arose that there might be a quantitative relationship between the reactions with the gold chloride and the strength of the toxin.

Graded mixtures of gold chloride (1 per cent. solution in distilled water) and diphtheria and tetanus toxins were, therefore, tried. Without considering at present the difficulties encountered in determining the optimum conditions, such as concentration of the toxin, etc., the result given in table 1 is representative of a preliminary titration carried out to determine the color range. If we were to utilze the procedure for quantitative comparisons of different toxins obviously an end-point would have to be found. Comparison of the reactions obtained with toxin-broth filtrates and with control-broth of similar constitution suggested that the green-color range was specific for the toxic broth filtrates. A representative titration within the green range is shown in table 2. The point of maximum green coloration was selected as the end-point.

The comparative results with a series of diphtheria and tetanus toxic broth filtrates are given in table 3. The method of titration was as follows:

A 1 per cent. watery solution of gold chloride was pipetted into a series of tubes in gradually increasing amounts (table 2). Then 2 cc. of the proper dilution of toxin were added to each tube. The tubes were shaken to mix the reagents, and incubated at 50°C. Readings were made at the end of forty minutes, and the amount of gold chloride giving the maximum intensity of green color noted. The first toxin in each series was tested on guinea pigs and the M.L.D. was accurately determined. On the basis of this toxin, the probable M.L.D. of the other toxins was calculated on the comparative amounts of gold chloride required to give the greatest intensity of green coloration. Their M.L.D. values were then determined by the guinea-pig test and a startlingly close parallelism resulted with most of the toxins, as may be seen in the table.

The L+ values of some of the toxins were also determined, but these showed no parallelism with the gold chloride values.

Two discrepancies in titrating for the M.L.D. values are shown in table 3. The titration procedure is subject to certain disturbing influences and these were probably the source of the discrepancies.

The main disturbing factors in the method are briefly as follows:

Overlapping of the color-zones sometimes occurs (see table 1). If other colors invade the green zone, the determination of the end-point may become uncertain. Some observations on the use of greater concentrations of sodium chloride in the diluent suggest that the resulting shift of the green zone to a point further removed from the other color zones may eliminate this difficulty.

TABLE 1.

PRELIMINARY TITRATION—TETANUS TOXIN 122

Toxin (Diluted 1:4), c.c. .... Gold Chloride (1 per cent. watery solution), c.c......	2 0.05	2 0.1	2 0.15	2 0.2	2 0.25	2 0.3
Green coloration	—	—	×	∓	—	—
Purple coloration*	—	—	—	—	∓	—
Blue coloration*	—	—	—	—	—	+
Flocculation*	—	—	—	—	+	+

The tests were incubated in a water bath at 50°C. for thirty minutes.

The diluent = 0.85 per cent sodium chloride in doubly distilled water.

*The sequence of purple and blue colors and the zone of flocculation varies in different lots of toxins.

++ = the most intense coloration; ∓ = very strong coloration; + = strong color; ± = weak color; × = trace; — = no coloration.

TABLE 2.

FINAL TITRATION—TETANUS TOXIN 122

Toxin (diluted 1:4), c.c Gold Chloride (1 per cent solution), c.c..	2 0.16	2 0.17	2 0.18	2 0.19	2 0.20	2 0.205	2 0.21	2 0.22	2 0.224	2 0.226	2 0.228	2 0.23	2 0.232
Green Coloration	×	±	+	+	+	∓	∓	∓	∓	++	++	++	∓

Flocculation occurs when a certain concentration of gold chloride is reached in the mixtures. When the zone of flocculation coincides with the zone of green color, the flocculate carries down the color with it, and accurate readings can not be made. The indications are, however, that the use of a greater concentration of sodium chloride will also eliminate this disturbing factor.

There are certain difficulties to be overcome in this method:

The optimum concentration is not the same with all lots of toxin. In most instances a dilution of 1 : 3 or 1 : 4 is satisfactory.

The quantitative range of gold chloride within which the green zone appears is so small that the green coloration may not occur unless close titrations are made. For the same reason great accuracy is required in measuring the reagents. Neutral glassware and doubly-distilled water are essential.

Several scarlatinal toxins have been tested with the gold chloride. A green zone occurred in all of them. The most that we may say at present is that the values calculated by this method and the skin-test-dose values showed sufficient similarities to suggest that the method could be employed for titrations.

TABLE 3.
COMPARISON OF COLORIMETRIC TITRATIONS AND GUINEA PIG TESTS

Toxin	Gold Chloride	Number of M.L.D.'s per cubic Centimeter—Undiluted Toxin	
		Gold chloride test	Guinea-pig test
	*grams**		
Diphtheria 696	0.0042	—†	175
Diphtheria 711C	0.0034	145	150
Diphtheria 717	0.0034	145	150
Diphtheria 718A	0.0046	195	200
Diphtheria 718B	0.0050	220	250
Diphtheria 720A	0.0039	165	175
Diphtheria 721B	0.0039	165	300
Diphtheria 722	0.0084	400	425
Diphtheria 8	0.0068	283	300
Tetanus 118	0.0045	—†	6,800
Tetanus 119	0.0029	4,382	4,500
Tetanus 120	0.00416	6,284	7,000
Tetanus 121	0.00424	6,400	10,000+
Tetanus 122	0.0046	7,743	7,800

*The tests were carried out with a 1 per cent solution of gold chloride in doubly distilled water, and 2 c.c. of toxin diluted 1:2, 1:4, or 1:8 with physiological saline. The results given in the table are calculated on the basis of 1 c.c. of undiluted toxin and the amount of gold chloride used in grams.

†Basis for calculation.

SUMMARY

By means of a colorimetric reaction obtained by adding gold chloride to toxic-broth filtrates we have been able to obtain toxin values which in most instances closely approximate the toxin values obtained by animal tests. Thus far we have not observed a colorimetric end-point which parallels the combining value (toxin-toxoid) of toxins.

We have no data as to the mechanism of the reaction. Whether the reaction is due to a specific toxin-gold chloride combination, or whether some other factor or factors are involved remains to be proven.

Whatever the mechanism of the reaction may be, the results strongly suggest that a practical, gold chloride colorimetric method of standardizing toxic-values of broth filtrates may be developed.

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[1] Osman, M. B.: Jour. Immunol., 1927, xiii, 243.

STUDIES IN INTESTINAL BACTERIOLOGY I

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Agnes Goldman

The object of this investigation was to ascertain the variations of the bacterial flora, both aerobic and anaerobic, in respect to numbers and to species, at certain levels of the small intestine, in health and disease.

A beginning has been made to discover whether such a study may yield any significant results in the investigation of obscure diseases, especially such as have, at various times, been ascribed to focal infections in the intestines or to toxic bacterial products of alimentary tract origin.

An intensive study of one person was made, with the purpose of observing the effect of special feeding on the intestinal and also on the fecal flora, and serologic methods were employed in order to throw further light on the question of an indigenous flora, or of a possible streptococcal focus of infection.

Conclusions

The following conclusions are arrived at from a study of the contents of the human small intestine, chiefly at the jejunal level:

The number of viable bacteria in the contents of a number of normal persons and hospital patients varied within wide limits.

The number of viable bacteria in the contents of diseased persons confined to a light meat-free diet usually appeared to be considerably higher in patients with gastro-intestinal disturbances or with diseases associated with metabolic disturbances or with focal infections of possible gastro-intestinal origin than in patients with diseases apparently unconnected with the alimentary tract.

The flora, in normal and pathologic cases, was usually of mixed bacterial character, forms, with widely varying metabolic requirements, multiplying in the same specimen.

Even when the contents were entirely dominated by one bacterial species, further analysis into cultural and biochemical properties revealed the presence of two or more varieties.

Several specimens from the same person taken under similar conditions usually showed approximate correspondence in bacterial numbers and in the predominant species.

Gram-negative bacilli of the colon group, gamma type streptococci, and gram-positive bacilli of the acidophilus group appeared to be a normal feature at the levels studied.

Spore-bearing aerobes, accompanied by proteolytic activity, appeared to be a normal feature of the intestinal contents.

Anaerobes were absent in four normal specimens but were found in many specimens from pathologic subjects, but not necessarily in all specimens from the same person. Large numbers of spore-bearing aerobes were not as common as in specimens from normal persons.

The presence in considerable numbers of spore-bearing aerobes we attributed in our normal subjects to the presence of meat in the diet.

This investigation has been made possible through the cooperation of Dr. Warren Coleman and Dr. William H. Park, and grew out of the investigations previously made at the suggestion of Dr. Coleman. The method of obtaining intestinal specimens had been developed and reported on by Aranovitch, Coleman and Einhorn. The expenses associated with the part time services of a physician and of the whole time of two laboratory assistants were defrayed by a fund, provided by New York University for Research Investigations in the Department of Medicine of which Dr. Coleman had the disposal. We wish to thank Dr. Park and him for valuable suggestions made to us in the course of the work.

We are also indebted to Dr. Curphey for obtaining the intestinal specimens and to Mrs. Betty Kolchin and Miss Helena Otto for their valuable assistance in the laboratory.

For bibliography see the original article.

STUDIES IN INTESTINAL BACTERIOLOGY II.

The Effect of Special Feeding on the Intestinal Flora

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Agnes Goldman

In the first paper in this series, I discussed the bacterial flora of the small intestine of 4 normal persons and of 20 hospital patients.

One of the female patients W. (arthritis deformans) was particularly ready to take the intestinal tube, and was most conscientious in following directions. She was kept at the hospital for several months, at the request of Dr. Coleman, receiving treatments in the form of bakings, massage and vaccine injection for the arthritic condition.

Altogether 20 intestinal specimens were obtained from her. The methods were the same as those described in the earlier paper. In the work with B. acidophilus we attempted to make use of whey agar plates as recommended by Rettger and Cheplin with but little success.

During much of the time she was receiving B. acidophilus culture (Arlington Chemical Co.), usually 6 teaspoonfuls a day. Later we isolated the strain from one of the bottles, and prepared with it cultures in dextrose broth. The strain isolated corresponded morphologically and culturally to descriptions of B. acidophilus, except for the fact that it did not ferment maltose. We do not know whether this strain never fermented maltose, and therefore did not correspond to the usual definition of B. acidophilus, except for the fact that it did not ferment maltose. We do not know whether this strain never fermented maltose, and therefore did not correspond to the usual definition of B. acidophilus, or whether it had lost this property.

The number of viable bacteria in the broth cultures, which were tested from time to time, varied considerably. The strain was extremely capricious. A fresh 18-hour culture had at least 200,000,000 viable bacteria per c.c., and sometimes several billion.

Table 1 is divided into two parts. In the first part are given the results while the patient was at the hospital on regular hospital diet receiving treatment and showing considerable improvement in her general condition. During most of the time she was getting 6 teaspoonfuls a day either of the commercial preparation called Bacid, or of our laboratory preparation. There is reason to believe that the culture which she was taking from June 20 to July 20, and which was of our own preparation, was largely nonviable, as it was probably kept too long.

All recent experiments agree that such small doses of B. acidophilus, especially if they are not reenforced with lactose feedings, are entirely ineffectual in changing the flora, and we may, therefore, look on this first set of records up to July 6 as observations under normal dietary conditions.

The first 4 specimens, taken over a period of 1 month, show greater variation in bacterial numbers than the earlier specimens. A count of 66,000,000 was far in excess of anything found in the previous specimens of this patient. No reason was found to account for the sudden increase in numbers or the predominance of gram-negative bacilli, which gave typical B. coli-communis sugar reactions, but it is possible that we here have an ex-

TABLE 1.

STUDY OF INTESTINAL CONTENTS OF W. OVER A PERIOD OF FOUR MONTHS

Date	Nature of Specimen	Feeding	Number of Bacteria per C.c. of* Intestinal Contents or of Fecal Suspensions on			Predominant Bacteria on Aerobic Pour Plates	B. acidophilus Group	Approximate Percentages# of B. acidophilus Group of Total Aerobic Count
			Endo Plates	Blood Veal Plates	Acid Beef Liver Plates			
Summary of Results Under Normal Conditions and After Special Feeding								
5/ 3	Intestinal, approximately 70 to 80″	....	10,200	1,200,000	3,240,000	Streptococci	+	< 1
5/19	Intestinal,† 70″	5/4-5/21: 6 teaspoonfuls of Arlco B. acidophilus culture daily	3,000,000	45,000,000	66,000,000	Colon group	+	< 1
6/ 2	Intestinal, approximately 50 to 60″	....	3,000	4,000	4,000	Streptococci	—	< 1
6/ 7	Intestinal, approximately 50 to 60″	6/3-6/13: 6 teaspoonfuls of Arlco B. acidophilus culture daily	< 20	7,200	2,400	Diplococci	+	< 1
7/ 6	Intestinal, approximately 45 to 50″	6/28-7/7: 150 gm. lactose daily.....	550,000	1,062,000 (80% streptococci)	1,457,000	Streptococci	+	< 1
7/14	Rectal swabbing	7/7-7/13: 100 gm. lactose daily.....	1,500,000	2,000,000 (37% streptococci)	5,300,000 (22% B. acidophilus)	Colon group	++	20
Summary of Intensive Feeding Experiments with B. Acidophilus and Lactose								
8/ 2	Intestinal, 90″	7/19-7/28: 6 teaspoonfus laboratory culture of B. acidophilus freshly grown every 3-4 days	600,000	6,000,000	9,400,000	98% streptococci	++	> 1
8/ 2	Rectal swabbing	7/28 and 7/29: 100 gm. lactose daily; 7/30 and 7/31: 50 gm. lactose daily; 8/1; no lactose	100,000	1,600,000	400,000 (30% B. acidophilus)	Colon group	++	5-10
8/ 5	Intestinal, 100″	8/2: 30 c.c. laboratory culture of B. acidophilus strain fed through 90″ tube; 8/3 and 8/4; no feedings	820,000	1,450,000	1,370,000	Streptococci	+	< 1
8/ 5	Rectal swabbing	....	1,600,000	1,700,000	1,300,000	Colon group	++	4-5
8/11	Intestinal, 100″† Some coiling in stomach, approximate position doubtful	8/5-8/7: no feedings; 8/8: 75 c.c of laboratory culture of B. acidophilus strain fed through 100″ of tube; 8/9, 8/10: no feedings	3,050,000	5,520,000	7,310,000	Streptococci	++	> 1.5
8/15	Intestinal, 80″	8/11: 75 c.c. of laboratory culture of B. acidophilus fed through 100″# of tube plus 100 gm. lactose; 8/12, 8/13, 8/14: 100 gm. lactose daily	< 20	13,400	13,600 (yeast)	Streptococci and sporing gram-positive bacilli	—	< 1
8/15	Rectal swabbing	....	109,200	108,400	268,000	Colon group	—	< 1
9/14	Intestinal, 65 to 70″†	8/22-9/15: 250 c.c. daily of laboratory culture of B. acidophilus strain plus 100 gm. lactose daily........	29,800,000	16,800,000	14,700,000	Streptococci and colon group	++	1.5
9/14	Fecal swabbing	....	85,000	125,000	148,000	Colon group	+	< 1

++, present in large numbers or amounts; +, present; —, not present as far as observed.

* The fecal suspensions from rectal swabhings probably represented between 2-10 milligrams of feces per c.c.

† The high counts on these intestinal specimens indicate that a reversed peristalsis may have taken place.

# The approximate percentage of B. acidophilus was estimated by noting the percentage of colonies present on the acid beef-liver plates and by noting the highest dilution of the N/10 acetic broth tubes in which morphologically characteristic bacilli developed.

ample of reversed peristalsis such as later occasionally was found to take place. The next 2 specimens showed an unusually low count. Another sample not included in the table, taken June 14, with an open olive, showed a count of less than 20,000 bacteria per c.c.; thus over a period of about 2 weeks, 3 successive specimens gave evidence of a decided reduction in bacterial numbers. Whether this reduction was related to the patient's generally improved condition or is attributable to the somewhat higher level from which the specimens came, we are not in a position to state. Some coiling of the tube was reported, and it was difficult to estimate the depth of the olive.

On June 28 she started taking lactose, 150 gm. per day until July 7, followed by 100 gm. per day from July 7 to 13. On July 3 a mild diarrhea set in. A specimen taken on July 6, shows a rise in bacterial numbers to over 1,000,000 per c.c.

The last specimen is a rectal swabbing taken at the close of the period of lactose feeding. A suspension in broth or salt solution was made from all swabbings. The amount of material was not weighed, as comparative rather than absolute numbers were desired. Counts on the different plates poured from the same rectal or fecal specimen give indications of the proportions of different bacterial groups. One c.c of suspension represents approximately between 2 and 10 mg. of feces.

A noteworthy point is the fact that all intestinal specimens, except the one with an excessively high count, show a predominance of streptococci. Even the rectal swabbing shows a fairly high percentage (37%) of green-producing streptococci.

We may note another related fact, that in the case of all the high count specimens the plates poured with acid beef-liver medium are by far the most crowded, even in the one with a predominance of gram-negative bacilli. This indicates that the flora of this patient was glycophilic and decidedly aciduric. The feeding of lactose encouraged these aciduric and glycophilic organisms both in the small and large intestine.

On investigating the proportion of B. acidophilus, one finds that, as might be expected with such an aciduric flora, this organism was usually present although in small numbers. It often overgrew all other organisms in the milk tube, and it was frequently isolated from the N/10 acetic acid broth. The approximate percentage of B. acidophilus-like bacilli of the total aerobic plate count has been put down as less than 1% whenever there were too few present to make an estimate, although in many cases the proportion is probably much under 1%.

Since the members of this group were normally present, we should expect the lactose to stimulate their development. From the intestinal specimen of July 6, we now find a few characteristic colonies appearing on the acid beef-liver plates, but on the whole the sugar is much more strongly favorable to the streptococci. The most crowded, however, of the acid beef-liver plates, poured from the rectal swabbing, showed 22% of B. acidophilus colonies. The acid beef-liver plates poured from earlier rectal specimens had no colonies of this type. We may say, at this point, that our search for B. acidophilus was thorough. We looked not only for the fluffy, but also for the smooth edged, types of colony; and we made many isolations and stained films from colonies whenever we were in doubt.

The second section of table 1 is given over to the record of an experiment in feeding a viable B. acidophilus culture through the tube, and then for two weeks administering large quantities per os, together with lactose. Our object in the first case was to avoid the action of the gastric secretion.

We intended to give the patient a certain length of tube to swallow, to take a specimen, and then before removing the tube, to inject some culture into the intestine. The patient was then to have a day of rest, and on the third day we were again to give her the tube to swallow with the purpose of having the olive reach a point below the level attained on the previous occasion. The object was to ascertain, through the intestinal specimen, and through a rectal swabbing, whether B. acidophilus had established itself below the point of injection. We then intended to follow the same method of procedure, administering culture and lactose simultaneously, the lactose, however, being taken by the mouth.

The chief difficulty was in controlling and confirming the point reached by the tube. According to the roentgen-ray reports, there seems to have been some coiling in the stomach on several occasions. Nevertheless had B. acidophilus become established, we might expect it to spread over a considerable region.

Besides pouring the acid beef-liver plates with 3 dilutions we also made dilutions of the specimens in a series of N/10 acetic dextrose broth, using from 0.5 c.c. of 1:10 to 0.1 c.c. of 1:10,000 or 0.1 c.c. of 1:100,000 dilution. Sometimes these tubes gave evidence of a growth which on the stained film morphologically resembled B. acidophilus, when no typical colonies could be found on the acid beef-liver plate. Often the bacilli in the N/10 acetic broth were nonviable—development was apparently initiated and then inhibited.

In obtaining the percentage of B. acidophilus, we made use both of the acid beef-liver plates and the N/10 acetic broth tubes. If morphologically characteristic organisms were found on the stained film from a N/10 acetic tube made up with 0.1 c.c. of a 1:1,000 dilution of the original material, while none was present in a tube containing 0.1 c.c. of a 1:10,000 dilution, we considered that between 10,000 and 100,000 B. acidophili were present per c.c. We then compared this number with the highest count on a plate having no B. acidophilus colonies. While it might be objected that many bacteria of different species which are too feeble to grow on a plate might develop in a liquid medium, and that therefore we were estimating our percentage of B. acidophilus too high, it is also true that N/10 acetic broth even with 2% dextrose is a severe medium, and that therefore a tube must be fairly heavily inoculated for growth to take place.

The two specimens, intestinal and rectal, taken on August 2, followed a week of B. acidophilus feeding (6 teaspoonfuls a day) and 3 days of lactose feeding. In the intestinal specimen over 1% and in the rectal specimen from 5 to 10%, of B. acidophilus was present. After stopping the lactose, and feeding 30 c.c. of culture directly through the tube, there was an apparent drop of B. acidophilus content both in the small intestine and in the feces. After feeding 75 c.c. of culture, there was apparently a slight rise of B. acidophilus in the intestinal content, but a further drop in the rectal content. August 15 another 75 c.c. of culture was injected through the tube, and on this and the following 3 days 100 gm. of lactose was taken. Neither the intestinal nor rectal specimens showed any B. acidophilus. Moreover, in none of the specimens was there a reduction of the amount of gas in the dextrose broth fermentation tubes. From this experiment, we conclude that a single injection of a concentrated B. acidophilus culture directly into the small intestinal tract is of no value in establishing the organism. The results might be different if the tube were retained long enough for several injections to be made. We wish, however, to call attention to the presence of free spores in the sediment of the dextrose fermentation tube (the record of observations on this medium is not included in the table), and to the predominance of yeast on the acid beef-liver plates of the intestinal specimen of August 15. Kendall observed that when animals are fed for some time on carbohydrates, gram-positive spore-bearing bacilli which slowly liquefy gelatin appear as a kind of intermediate adaptive flora. The spores in the sugar tube and the yeast seem to us to represent a type of intermediate flora appearing in response to a few days of lactose feeding.

Finally, we fed our patient for 2 weeks daily with 250 c.c. of a laboratory culture in dextrose broth of B. acidophilus, together with 100 gm. of lactose. We then had every reason to expect an implantation. After this period the intestinal contents showed between 1 and 10% of B. acidophilus-like bacilli in the N/10 acetic broth, and no typical colonies on the acid beef-liver plates. We again note the great increase in total numbers observed in a single previous specimen. From a fecal swab we made an estimate of less than 1%. This gave lower results than had been given by a previous feeding with lactose alone. A fecal swabbing taken 4 days after the feedings were stopped showed about 5% of B. acidophilus colonies.

The question naturally arises whether the strain used by us, derived from a commercial bottle, was adapted to implantation. We stated at the beginning that this strain had lost, if it ever had possessed, the faculty of fermenting maltose, which is supposed to be characteristic of B. acidophilus. In this connection we wish to state that every strain resembling B. acidophilus isolated either from the acid beef-liver plates, milk or acetic broth tubes, and tried on maltose, invariably fermented this sugar, some strains more slowly than others. From this result we must conclude that there never was any implantation of the strain administered, and that fluctuations in the percentage of B. acidophilus were entirely due to lactose feedings or to some other fortuitous factor. Work carried on in our laboratory by Miss Otto indicated that with several other strains also true implantation did not take place.

Experiment with Sterile Food

The patient left the hospital and returned to her home on Oct. 1, 1922. November 20, she came late in the evening to the Willard Parker Hospital, where she was under our direct observation. That night she took a cathartic. From the following morning she was served with food that was as nearly sterile as possible. She received sterile water, boiled milk, oranges, boiled eggs, and thoroughly toasted bread. Her dishes were kept apart and boiled. She washed her hands thoroughly and gargled with a disinfectant before eating. The only nonsterile food she received was 100 c.c. of a laboratory culture of the same B. acidophilus strain which she had previously taken. On Nov. 22 the tube was passed, and on the morning of the 23rd a specimen was taken. During this entire period her food was controlled.

The roentgenogram giving the position of the tube, of which 75″ were swallowed, showed that no coils were present.

Great difficulty was encountered in aspirating any material. It was finally necessary to inject about 20 c.c of salt solution, followed by some air, to expel it from the tube, and then after waiting from 5 to 10 minutes, to aspirate again. By doing this twice, enough material for cultivation was obtained.

With the exception of 3 surface colonies of staphylococci regarded as contaminations, three plates, each of which contained 0.05 c.c. of the original material, were entirely sterile. So also was the dextrose broth fermentation tube, and the N/20 acetic dextrose broth. In the sugar-free broth fermentation tube we found a small diplococcus. A similar organism and a few small gram-positive bacilli were found in the stained film from the lowest dilution tube of the cooked meat medium. In the milk, which slowly coagulated, we found a minute, long chain streptococcus, 15 to 18 faintly gram-positive individuals constituting a chain. On cultivation, this strain formed minute colonies of the gamma type on a blood veal plate.

A first glance at the result of this experiment inclines one to the opinion that the bacterial flora of the small intestine may be determined by the bacterial features of the ingesta. There are, however, several reasons why such a conclusion seems unwarranted.

First, we must remember that several months had passed since the patient was discharged from the hospital, and that her general condition was much improved over what it had been during the first months after entering the hospital. She had not been taking any lactose, which, as we have seen, had a stimulating effect on the normal intestinal flora. Some recent experiments, not recorded here, have given confirmatory results, that is, they indicate a great natural reduction in the numbers of the intestinal flora.

Another important point is that in our effort to give her sterile food, we simplified her diet greatly. Except for a little bread, the food was liquid or semisolid, and for the most part easily digestible. Probably a large part of it was absorbed in the upper levels of the small intestine, and what remained passed rapidly into the colon without offering a nidus for the breeding of bacteria, such as would be afforded by masses of undigested meat or of carbohydrates.

The fact that the only organism successfully recovered was a delicately growing streptococcus, a member of the group most characteristic of the intestinal contents of this patient, would seem to suggest that here we have a representative of what, for this person, constitutes a part of an obligate indigenous flora. In the later experiments, just referred to, it was again found that the only recoverable organisms in two apparently almost sterile specimens were streptococci, this time of the alpha type, developing best in the milk tube.

Finally, the fact that the large numbers of viable acidophilus bacilli ingested were not recovered denotes that they passed directly out of the small intestine, without even temporarily establishing themselves.

Thus, it would seem to us that while bacteria introduced with the food or saliva may temporarily multiply in the interior of undigested masses of food, the bacterial numbers will rather depend on the amount and the nature of the diet than on its microbal constituents, while the basic flora, like that of the upper respiratory passages, will maintain itself through considerable environmental variations. It seems possible, however, that the rapidity with which the bacteria are eliminated may depend on the substratum on which they are introduced. Those in a liquid medium might be rapidly disposed of through the action of peristalsis, while those embedded in a more solid substance might multiply, especially in the presence of sluggish peristalsis, until they temporarily overshadowed or at least modified the picture of the permanent flora.

A similar series of experiments, over a protracted period, should of course be carried out on one or more normal persons, in order to determine whether a certain bacterial type is always predominant in spite of a variety of environmental changes.

SUMMARY

The strain of B. acidophilus fed in large quantities to one patient could not be implanted in the intestine. Lactose feeding was more effective in increasing the number of B. acidophilus. It seemed most effective, however, in increasing the number of streptococci.

Large numbers of viable B. acidophilus introduced directly into the small intestine were rapidly eliminated.

After a diet sterile except for a broth culture of B. acidophilus the intestinal content at a depth of 75″ was almost sterile, streptococci being the only recoverable organisms in a few tubes of liquid medium.

It is suggested that streptococci may represent the indigenous bacterial flora of this person at the level tested.

For bibliography see the original article.

STUDIES IN INTESTINAL BACTERIOLOGY III

A Study of Nonhemolytic Streptococci from Human Intestinal Tract.

Published in The Journal of Infectious Diseases, 1924, Vol. XXXIV, pp. 509-515

Agnes Goldman

Investigations on the antigenic relationships of the Streptococcus viridans group from human throats, have demonstrated its heterogeneous character. From the same person at a given time, two or more culturally similar but antigenically distinct strains may be isolated.

It seemed of interest, therefore, to examine the streptococci isolated from the intestines of one person at the same and at different times. Opportunity for such a study was afforded by material obtained through the Einhorn tube from patient W. in the manner described in the previous papers of this series.

The existence of similar immunologic reactions in two or more strains isolated at different times might throw some light on the question of a native or permanent flora in the small intestine. When the patient, as was the case with our subject, is suffering from arthritis deformans, often ascribed to focal streptococcal infection, the frequent reappearance of the same strain might indicate a possible relationship of the strain to the disease.

N. and J. Mutch state that there is a great increase in the absolute and relative numbers, and in the varieties of intestinal nonhemolytic streptococci in certain conditions of disease. Holman observes that focal areas of streptococcal infection often contain more than one type of streptococcus. There is also evidence that when green-producing streptococci appear in the throat associated with colds or influenza, a greater variety of strains is present than is found in normal throats.

The varieties isolated ranged from strains growing luxuriantly to those growing sparsely on ordinary dextrose agar, although none seemed as delicate as two strains obtained from a nasopharyngeal swabbing of the same patient. Some were of the gamma type; others were of the alpha type and produced a vivid green zone, followed by marked clearing after standing at room temperature, but most strains occupied an intermediate position, only slightly discoloring the blood, and developing a small hemolytic zone. Isolations were made from different mediums as indicated in table 1, which gives immunologic relationships, and sugar reactions of a number of strains derived from 9 intestinal and 1 nasopharyngeal specimen.

If one first examines the sugar reactions, it is seen that the intestinal streptococci fall into several groups. As might be expected, Strep. fecalis was isolated on several different occasions. Strep. equinus reactions were given by all strains tried on sugars from examination 4 (strains 11-14). They were originally rather delicately growing organisms. Strep. mitis, which, according to Holman, is the most common of the nonhemolytic strains, was recovered from 4, possibly from 5, specimens. Occasionally a sugar reaction was so faint (∓) that without repeating the test it would be difficult to classify the strain. Strep. ignavus was twice isolated. Strep.

TABLE 1

AGGLUTINATION AND SUGAR REACTIONS OF STRAINS OF STREPTOCOCCI ISOLATED FROM INTESTINAL SPECIMENS AND NASOPHARYNX OF W.

Cultures			Agglutination Reactions with Serum of Rabbit Immunized with						Sugar Fermentations					Classification According to Holman
			Strain 1		Strain 11		Strain 9							
No of Strain	No. of Specimen	Medium	Titer	Control Serum 1:100	Titer	Control Serum 1:100	Titer	Control Serum 1:50	Dextrose	Lactose	Mannite	Saccharose	Salicin	
1	1	Blood	1,600	0	0	0								
2	1	Blood	0	400	400	100	600	50	++	++	±	++	++	Mitis or fecalis
3	1	Cooked Meat	200	0	0	0	0	0	++	++	++	++	++	Fecalis
4	1	Endo	200	0	0	0	0	0	++	++	++	++	++	Fecalis
5	1	Endo	1,600	0	0	0								
6	1	Acid beef liver	800	0	0	0	0	0	++	++	—	++	++	Mitis
7	2	Blood	0	0	0	0								
8	2	Blood	0	0	0	0	0	0	±	—	—	—	—	Ignavus ?
9	3	Blood	0	0	1,400	100	600	0	++	±	—	±	++	Equinus or mitis
10	3	Blood	0	0	0	0								
11	4	Blood	0	0	1,400	0	0	0	++	—	—	++	++	Equinus
12	4	Blood	100	0	100	0								
13	4	Blood	0	0	1,400	0	0	0	++	—	—	++	++	Equinus
14	4	Blood	0	0	200	0	100	0	++	—	—	++	++	Equinus
15	5	Cooked Meat	0	0	0	0	0	0	++	++	++	+	++	Fecalis
16	10	Acid beef liver	1,600	100	0	0	0	0	++	++	—	++	++	Mitis
17	10	Acid beef liver	0	0	0	0	...	...	±	—	—	++	—	Ignavus
18	15	Blood	200	0	0	0	600	0	++	++	—	±	++	Mitis
19	15	Blood	0	0	400	0	0	0	++	++	++	++	++	Fecalis
20	19	Blood	.....	...	0	0	0	0	++	++	—	++	—	Salivarius
21	19	Whey	....	...	.....	..	0	0	++	++	—	++	++	Mitis
22	19	Acid beef liver	.....	...	.....	.	0	0	++	++	—	++	±	Salivarius or mitis
23	25	....	....	..	.....	.	0	0	++	++	++	++	++	Fecalis
24	Naso-pharynx	....	..	0	0	0	0	0	++	++	—	+	—	Salivarius
25	Naso-pharynx	....	1,600	0	0	0	0	50	++	++	++	++	++	Fecalis

The following symbols are used to indicate sugar reactions: ++, marked fermentation; +, faint fermentation; ±, doubtful fermentation; —, no fermentation;
aThe titer of a serum for any strain is the highest dilution showing complete or almost complete agglutination.

salivarius was isolated from one intestinal specimen only. Particular reference is made to this as it was unique.

The lower dilution blood plates from the original specimen showed a mixed flora, but the highest dilution plate was exclusively seeded with colonies of streptococci, which were eventually found to constitute over 98% of the total flora. One further analysis, the streptococci from this sample fell into two groups: one, represented by strain 21, formed short chains in broth, and moderately green-producing colonies on blood agar; the other, represented by strain 20 from a blood plate and strain 22 from an acid beef-liver plate, was found as a lance-shaped diplococcus that only after several transplantations began to form any chains in broth. The colonies on blood agar were minute, surrounded by a relatively wide vivid green zone. They resembled much more nearly the characteristic alpha type found in the throats of patients with cold and influenza. It is, therefore, noteworthy that they gave the sugar reactions of Strep. salivarius. This group is supposed to be found rarely in the intestine, but is often associated with focal areas in the nasopharynx. Two strains from this source (strains 24 and 25) gave the sugar reactions of Strep. salivarius, although they continued, even after long cultivation, to grow much more delicately than the strains 20 and 22 from specimen 19. The single strain of Strep. salivarius isolated by Holman from the feces was a long chain variety, in this respect differing from ours. We tried repeatedly to isolate a similar type from subsequent specimens, but were not able to recover a strain culturally resembling the diplococcoid form of strains 20 and 22. As is well known, Poynton and Paine found a diplococcus which they thought was directly associated with rheumatoid arthritic conditions.

If one now turns to the cross agglutinations which were carried out with serums from rabbits immunized against strains isolated from the first, third and fourth intestinal specimens, one finds that serum of strain 1 partially agglutinated all of the strains from the same specimen, except strain 2. It also slightly agglutinated a few strains from later specimens. Some of the strains partially agglutinated by the serum may be more closely related to the immunizing strain than appears from the result. It is probable that the agglutination in higher dilution of the immunizing strain may be due to its constant transplantation during the period of injection. Nevertheless, there are few strains that are completely agglutinated by the serums even at the dilution of 1:100. The only "fishing" completely agglutinated by serum of strain 1 was a streptococcus fecalis of nasopharyngeal origin. The two strains strongly resembled one another in morphologic and cultural characteristics. Unfortunately absorption tests could not be made because strain 1 died out during the course of the work. The relationship is suggestive, but no definite conclusions can be drawn without the confirmatory test.

Serum of strain 11 strongly agglutinated one strain from the same specimen and none from any other specimen. Serum of strain 9 was not tried on any strains from the same specimen. It strongly agglutinated one strain from specimen 15, i. e. strain 18. In order further to investigate the relationship between the two strains, an absorption test was carried out. It was found that strain 18 completely absorbed the specific agglutinins for strain 9. A rabbit was then immunized with strain 18 and reciprocal agglutination and absorption tests were made. It then appeared that strain 9 absorbed all the specific agglutinins for 18. Thus by the most delicate test at our disposal the identity of the two strains was established.

The data of the absorption tests are given in table 2. The method followed was that used in our serologic study of alpha streptococci from the throat.

TABLE 2.

AGGLUTINATION BY SERUMS 9 AND 18 BEFORE AND AFTER CROSS ABSORPTION

	Strain 9	Strain 18
Serum 9	1,000	1,000
Serum 9 treated with strain 9	0	0
Serum 9 treated with strain 18	0	0
Serum 18	1,000	1,000
Serum 18 treated with strain 9	0	0
Serum 18 treated with strain 18	0	0

We conclude from this set of experiments that there must be present in the upper intestinal tract of W. at different times a considerable variety of streptococcal types. Nevertheless, the fact that we have isolated, at intervals of 2 months, 2 strains apparently identical, may indicate either that there is a flora which is more or less permanently established in the intestinal tract or that there is a focus at some other point from which these organisms are introduced into the alimentary canal.

While it is of course quite possible that one streptococcal strain among many in the tonsils, teeth or intestinal tract may be directly responsible for the symptoms of disease, the mere presence of an unusual number and variety of nonhemolytic streptococci hardly justifies the assumption of a causative inter-relationship of any one or all of them to the pathologic condition, especially in view of the fact that these bacteria are common secondary invading organisms. N. and J. Mutch suggest that "a negative phase in the immunity reactions of the body, determined by increased bacterial activity at one focus, affords bacteria of allied race, opportunity of gaining simultaneously advantage over the cells of distant tissues among which they live."

In view however, of the recognized adaptability to mucosal surfaces of the nonhemolytic streptococci, the establishment of members of this group in the small intestine does not necessarily seem to imply the existence of a pathologic flora, any more than does the extreme predominance of the colon group at lower levels of the intestine. It seems plausible to regard the streptococci, when present in large numbers, as indications of deranged intestinal functioning or of excessive amounts of carbohydrate in the diet rather than as evidence of the existence of a primary focus of infection with production of toxic substances or with tissue invasion. Lactose feeding, we found, stimulated this group, and it has been affirmed that the presence of carbohydrate associated with loci of stagnation is the most potent factor in establishing its predominance just as undigested proteins are supposed to favor the proteolytic organisms. It seems probable that an adaptation of particular bacterial types to the metabolic conditions within the tract of each person may gradually develop, until certain individual strains become indigenous.

Several strains of intestinal streptococci were tested for direct agglutination by the serum of the patient W. using normal serum as control.

As the patient had some months earlier received an autogenous vaccine of intestinal streptococci, positive reactions were expected (table 3).

TABLE 3.

AGGLUTINATION TESTS BY WALSH AND GOLDMAN SERUMS OF STRAINS OF INTESTINAL STREPTOCOCCI

Number of Strain	Serum	Titer
20	W	3,200
	G	1,600
11	W	100
	G	100
18	W	3,200
	G	400
9	W	3,200
	G	1,600
4	W	100
	G	100
25	W	0
	G	0
Burke	W	1,600
	G	800

Agglutinations were carried out on 2 separate days; the first set was carried through a 1:100 serum dilution. The second set was carried out with 1:100 as the lowest serum dilution.

In the first test, we used 1:100 as the highest dilution, but when it was found that in all lower dilutions a prezone developed, we repeated the test with higher dilutions, using in the case of the patient some serum that had been held over for 3 days.

It was found that both the patient's and the control serum agglutinated most of the strains in high dilution. The patient's serum was the more potent, but the difference seems of little significance. This was the case not only when the serum was tested with autogenous strains, but also when tested with a strain isolated from the specimen of another subject (B). The test with the streptococcal strain of naso-pharyngeal origin was negative. Unfortunately it was not possible to test it with as high a dilution of serum as was used for the other strains.

These results prove how misleading the mere fact of a high titer agglutination may be. They also indicate that since human serum appears to have developed a high antibody content against intestinal streptococci, these organisms must be regarded, as indeed the flora of the feces would lead us to suppose, as normal inhabitants of the intestinal tract.

Summary

The same strain of nonhemolytic streptococcus, identified by agglutinin absorption methods, was twice recovered from the intestinal contents of one patient, at an interval of two months.

The persistence of this strain may indicate a well adapted flora, native to the intestinal tract of the person from whom the strain was isolated or a focus at a higher level from which the strain was at intervals disseminated.

Normal human serum appears to contain a high antibody content of group agglutinins for some nonautogenous streptococci from the small intestine.

For bibliography see the original article.

HYDROGEN ION CONCENTRATION.

A brief and simple discussion of the meaning of pH and a modification of Medalia's method for its determination in the phenol red range.

Jacob Weinberg

Introduction

In the last ten years the "hydrogen ion concentration," the measurement of acidity by the so-called pH, has constantly been gaining in popularity. It is now being extensively applied in numerous industries and especially in the various branches of the biological science. Quite frequently, therefore, such tests have to be performed by workers who cannot afford to go into the details of the theoretical considerations of the pH, for these as treated in the modern works on physical chemistry involve higher mathematics and principles of a complicated nature. An elementary simple and brief discussion of hydrogen ion determination will in many cases be helpful. For this reason before commencing the treatment of the problem an attempt will be made to touch upon some fundamental points related to hydrogen ion concentration. One of these is the definition of an acid and a base.

An acid is defined as a substance which when in solution

1. Turns blue litmus red;
2. Gives a sour taste;
3. And reacting with a base yields a salt:

   or
4. Dissociates into its components, one of which is hydrogen ion.

All of these definitions, though stating the true qualities of an acid, are nevertheless, insufficient in the light of the new theory of ionization.

The first one implies an indicator and since not all of the many indicators respond to the same degree of acidity or alkalinity, the impracticability of such a definition is obvious. A substance may be acid to litmus and alkali to methyl red, alkali to litmus and acid to phenolphthalein. Furthermore this definition does not take care of the transitional value, i.e., of the value for instance when the color changes from a distinct blue to a distinct red in case of litmus and vice versa.

Disregarding the taste property for an obvious reason and characterizing the third definition as indefinite because precisely as the acid is defined in terms of a base so is a base defined in terms of an acid, we may pass on to the fourth. This one sounds more scientific and appears to be more correct than the others. It is, however, very far from being true, for the most concentrated aqueous solutions of bases contain some hydrogen ions (Clark, p. 21). This consideration follows from the equation.

$$[H^+] \times [OH^-] = Kw$$

$[H^+]$ = hydroxide ion concentration
$[OH^-]$ = hydroxide ion concentration
Kw = constant for water

In order to satisfy this equation, one of the variables must increase when the other decreases but neither one in an aqueous solution can ever be reduced to zero because Kw is constant and greater than zero. The mere assertion then that a solution containing hydrogen or hydroxide ion is an acid or a base, respectively, is not sufficient for the identity of the same. If to this quality factor a quantity factor be added, the resulting definition will be satisfactory. In other words, we must arbitrarily assume some definite amount of hydrogen ion for the neutrality point; an increase in this value will cause acidity; a decrease, alkalinity. Such an arbitrary number is suggested by the above equation. The constant Kw was found to be approximately 1 : 100,000,000,000,000 which means that in a liter of pure distilled water the product of $[H^+]$ by $[OH^-]$ is always approximately equal to $1:10^{14}$. Since in the case of the dissociation of water for every hydrogen ion there must be a hydroxide ion and the factors on the left side of the equation are equal, the number on the right side of it is to be resolved into two equal factors also. $1:100,000,000,000,000 = 1:10,000,000 \times 1:10,000,000$ *or* $1:10^{14} = 1:10^7 \times 1:10^7$. It is hereby proposed then that the quantity $1:10^7$ may be introduced into the definition of an acid: An acid is a substance which when dissolved in water yields a greater concentration of hydrogen ion than $1:10^7$ gms. per liter; a base is a substance which when dissolved in water yields a smaller concentration of hydrogen ion than $1:10^7$ gms. per liter. In other words the difference between an acid and a base is not of kind but of degree, depending not on the mere presence of hydrogen ion but on a definite amount of it.

The definition was dwelt on at length because it is the basis for a better appreciation of the importance of H-ion determination and for a clearer understanding of the meaning of a pH. This shall presently be discussed briefly.

A normal acid is one which contains in a liter of solution one gram of ionizable hydrogen. It makes no difference whether the acid is monobasic like HCl, dibasic like H_2SO_4, or tribasic like H_3PO_4, a normal solution of it should contain one gram of hydrogen in one liter of solution.

A. $\frac{N}{1}$ (Normal) 1 gm. H per liter $\frac{1}{10^0}$

B. $\frac{N}{10}$ (tenth normal) $\frac{1}{10}$ gm. H per liter $\frac{1}{10^1}$

C. $\frac{N}{100}$ $\frac{1}{100}$ gm. H per liter $\frac{1}{10^2}$

D. $\frac{N}{10,000,000}$ $\frac{1}{10,000,000}$ gm. H per liter $\frac{1}{10^7}$

E. $\frac{N}{100,000,000,000,000}$ $\frac{1}{100,000,000,000,000}$ gm. H. per liter $\frac{1}{10^{14}}$

For convenience we use the exponents of the denominators in combination with the symbol pH (potential hydrogen) to denote the corresponding normalities, that is, the pH value is equal to $\log \frac{1}{[H+]}$.

Thus the normality in line A is denoted by pH0
" " " B " " " pH1
" " " C " " " pH2
" " " D " " " pH7
" " " E " " " pH14, etc.

In accordance with our definition of an acid pH7 denotes a neutral solution, pHO, a normal acid and pH14, a normal alkali.

The pH value of a solution is determined electrometrically and colorimetrically. In the following pages only the colorimetric methods will be dealt with, not in all their details, but only in so far as to point out some discrepancies resulting from the recommendations of the several authors.

Comparison of Colorimetric Methods

There are three principal systems which are being used at present in the colorimetric method for the testing of the concentration of hydrogen ion in culture media. First, the Walpole system, which comprises the range of pH 2.7—6.5 and which consists of a mixture of standard solutions of acetic acid and sodium acetate. Second, the Sorensen standard phosphate solutions system which covers the pH range from 5.8 to 8.4. This range is the most useful as far as culture media are concerned since it is within this range that most of culture growth is possible. The standards are prepared by mixing definite amounts of dibasic sodium phosphate and monobasic potassium phosphate solutions. Third, the Medalia system, which even though not thoroughly scientific as the other two, is nevertheless of great importance on account of the large range of pH of which it is taking care (1.2—9.6) and on account of the comparatively little difficulty encountered in the preparation of the standard solutions. These are made up of hydrochloric acid and sodium hydroxide of known percentage or normality.

It is obvious that if any systematic work and accurate results are to be obtained, the pHs of the three systems especially at the overlapping points. should coincide. Not only is this coincidence necessary when a medium is tested before and after autoclaving, where the pH value falls or rises to such a point that two systems must be used for the two tests, but it is also of great importance when a comparison of tests or standardizations, done in different laboratories, is to be made.

In undertaking this work it was sought to establish first, where variations or deviations occurred and then if possible to so alter the standards as to make the systems agree. One of the systems, the phosphate solutions, was standardized against a potentiometer and these solutions in turn were used as standards for comparsion with the other two systems.

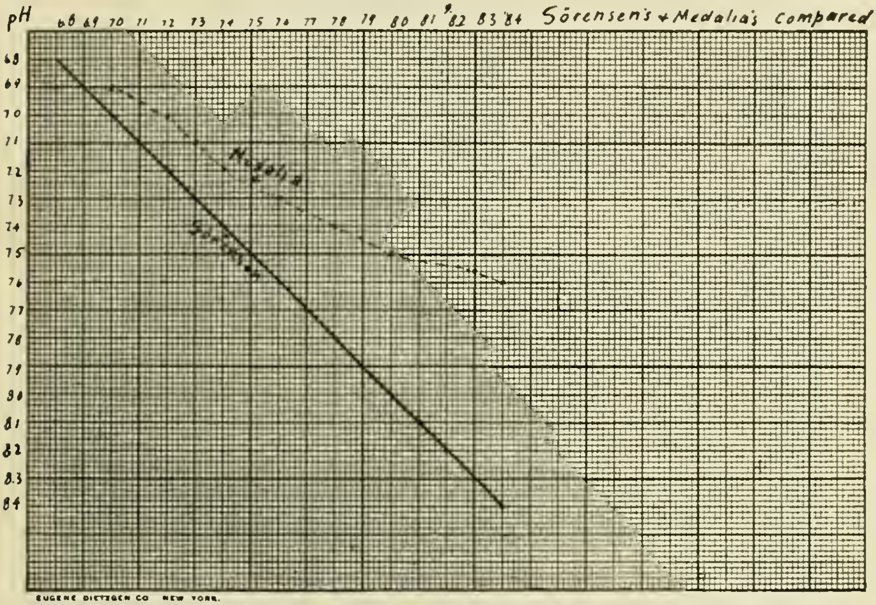
As regards the indicator used for the estimation of the pH, it should be pointed out here that there is a disagreement among the authors as to the percentage of the phenol red in solution and as to the solvent to be used in making up the indicator solutions. Medalia recommends 0.04% solution of phenol red for the phosphate standards; Park & Williams recommend 0.02% solution of phenol red. In this laboratory a 0.04% solution for both the Medalia and Sorensen systems has been in use.

A set of standard tubes of the range pH (6.8—8.4) were made up in each of these two systems. Different media of various pH values were tested against both systems under the same conditions of temperature, quantity, concentration of indicator, age of standards, etc. About one hundred tests were made but only sixty recorded. Only the averages of the reading are given below.

TABLE 1.

Medalia	Sörensen
pH	pH
6.9	6.8
6.9	6.9
6.9—	7.0
6.95	7.1
7.0	7.2
7.1	7.3
7.2	7.4
7.2	7.5
7.3	7.6
7.35	7.7
7.4	7.8
7.4	7.9
7.5	8.0
7.5—	8.1
7.5—	8.2
7.55	8.3
7.6	8.4

From an examination of this table it can be shown that not only do the values differ widely but the difference increases directly as the pH values. In the accompanying graph the values of the two systems are plotted against each other. The straight line represents the normal comparative values while the broken line shows at a glance the abnormal conditions. It was observed, however, that the end of the broken curve is midway between the ends of the horizontal line and the normal curve.



Graph 1—Showing comparative values by Sorensen's and Medalia's systems.

Now, since in the Medalia system only the amounts of indicator are varied while the solutions are of the same strength and amount in each tube, why should it not be possible to halve the intensity of color and make it conform to that of the Sörensen system by simply halving the quantities of the phenol red indicator solution? The affirmative answer to this question was verified by experiment. In several preparations made in this manner the expected results have become true. The equivalence of the intensities of the colors in both systems was proved beyond question.

The restoration of Medalia's standards was the result of this investigation. It resulted also in a great improvement upon this system which proves to be economical, saving time and labor and more reliable as far as accuracy is concerned. A glance at the following tables will aid in seeing the improvement.

TABLE 2

As recommended by Medalia (0.04 %) phenol red solution.

	6.8	7.0	7.2	7.4	7.5	7.8	8.0	8.2	8.4	
Amounts of 0.04 % phenol red alcoholic solution in each tube ...... cc.	0	.05	.1	.15	.2	.25	.3	.35	.4	Each tube contains 10 cc of $\frac{N}{100}$ Na OH
	.4	.35	.3	.25	.2	.15	.1	.05	.0	Each tube contains 10 cc of 0.1 % HCl*
SUM	.4	.4	.4	.4	.4	.4	.4	.4	.4	

*This is made up by simply mixing 1 c. c. of concentrated HCl with 999 c.c. of water. It is not a true % solution. It is about .037 %.

TABLE 3.
MODIFIED AND IMPROVED 0.04% SOLUTION

	6.8	7.0	7.2	7.4	7.6	7.8	8.0	8.2	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8	10.0	10.2	
Amounts of 0.04% phenol red alcoholic solution in each tube	0	.025	.05	075	.1	125	.15	.175	.2	.225	.25	.275	.3	.325	.35	.375	.4		Each tube contains 10 cc. of $\frac{N}{100}$ Na OH
	.4	.375	.35	.325	.3	.275	.25	.225	.2	.175	.15	.125	.1	075	.05	.025	0		Eachtube contains 100 cc. of 0.1% HCl
SUM	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4		

Notice, in examining tables 1 and 2 that the pH range was doubled for the phenol red indicator. Instead of the three indicators previously used for the range 6.8—9.6 which use was a cause of some of the discrepancies and irregularities, we are now enabled to use only one indicator with a uniform gradation and for a more extended range. This was made possible by halving the differences of amounts of indicator added to two successive alkali tubes.

It should be noted here that a little percussion, a little warming and shaking will help in dissolving the phenol red powder. Also, only small amounts of this should be made up at a time because it gradually loses its coloring power. New standard tubes with two weeks old indicator were found to have less color than the 2 weeks old standard tubes with the same indicator. The change must have occurred in the indicator solution not in the standard tubes. Another very important precaution is to keep the stoppers of the standard tubes absolutely dry. A stopper moistened by the solution in the tube will cause a change in color in a very short time. This has been here amply verified.

Conclusion:—Medalia's system cannot be used without a radical change in it. As recommended by Medalia without improvement the system must be condemned. Clark in his "The Determination of Hydrogen Ions," p. 131, calls Medalia's method misconceptions of theory and practice. Gillespe says: "The proposed tables (Medalia's) are incorrect, . . . the plan on which they are based lacks a solid foundation and it is not supported by enough data." Here they were proven to be in complete disagreement with the other reliable systems. However, it is advantageous and practical to use the standards of Medalia *with the modifications as here recommended;* (1) on account of the fact that for some reason the phenol red standard tubes keep their color for a very much longer period in the case of hydrochloric acid and sodium hydroxide than in the case of phosphates; (2) because of the extended range of pH in this system; and (3) because of the possibility of using a single indicator instead of three or four. Consequently the attempt was made to adjust it by varying the amounts of the indicator from what had been recommended by Medalia. The standard modified tubes are now in complete agreement with the other standards, at least between the values 6.8—8.4 and probably correct up to 9.6.

THE ELIMINATION OF THE PRECIPITATE FROM PHOSPHATE BROTH MEDIUM.

Jacob Weinberg

When phosphate broth medium is prepared according to the formula devised by our laboratory following the general directions of Dochez, Avery and Lancefield a precipitate invariably appears in the medium after autoclaving regardless of the elaborate filtering and regardless of the fact that after such a filtering but before sterilization the medium appears to be very clear.

The precipitate thus appearing has been reported to us by several visiting bacteriologists as well as by our own serologic workers as a great nuisance in antigen work where this medium is mostly used.

A very slight and simple modification has given excellent results in about forty preparations. Where the results were not perfect some deviation from this modification was found to be the cause. There are three points to be observed in this modification. First the bulk of the medium before it is filtered should be autoclaved; second such autoclaving should be at a minimum pressure of 20 lbs. for 30 minutes; and third the final sterilization should be under a *MAXIMUM* pressure of 15 lbs.

For the benefit of those who desire to have the details of the preparation of the phosphate broth a complete formula is here introduced.

Infuse chopped lean beef, which has been freed from fat and tendon, in the ice-box over night. One pound of beef in a liter of water will yield a good infusion. This is boiled until coagulation takes place. A few minutes is sufficient. Strain over cheese cloth. Add peptone (Fairchild's) to make one per cent. solution. Add sodium phosphate to make a 0.2% solution. Heat until all the peptone is dissolved. Adjust the reaction to pH 8.2; 33 c.c. of normal sodium hydroxide per liter of medium is the usual amount required for this adjustment. Heat the entire bulk of the medium in the autoclave under a pressure of 20 lbs. for 30 minutes. This rather high pressure is necessary to throw out at this stage all the precipitable material. If this is not followed precipitation will take place in the final sterilization and cause the endless trouble and interference with the serologic tests already referred to above.

The medium is now filtered through cotton and paper two or more times, depending upon the kind of paper used, and the reaction again adjusted to pH 7.8 by adding one c.c. of normal medium hydroxide per liter for every 0.1 pH lower than 7.8. It is important that the media shall be hot when filtered. The clear medium is bottled and sterilized for 30 minutes at a pressure of *not higher than 15 lbs.* The medium thus prepared ought to have no sediment or cloudiness whatever. The final pH should be 7.5 but 7.6 or 7.4 is satisfactory.

UNSUCCESSFUL ATTEMPTS TO CURE OR PREVENT TUBERCULOSIS IN GUINEA PIGS WITH DREYER'S DEFATTED ANTIGEN.

Published in The American Review of Tuberculosis, 1926, Vol. XIII, pp. 114-123

Mary Nevin, Florence R. Bittman and Elizabeth Lee Hazen

When Dreyer's work on the therapeutic value of a "defatted" vaccine, made from the *Bacillus tuberculosis*, was published, it created quite an interest in the medical world, which looked forward to a more extensive repetition of the work by numerous investigators and a possible verification of all that Dreyer had claimed.

On account of the fact that Dreyer had used so few animals, and, according to our opinion, insufficient controls, we decided to repeat his experiment, using a greater number of animals and more controls, and at the same time to test the prophylactic as well as the therapeutic value of the vaccine.

Summary of Results

According to our experiments, the defatted vaccine prepared according to Dreyer's method produced no beneficial results in tuberculous guinea pigs when treated either therapeutically or prophylactically. On the contrary, the animals treated with the vaccine succumbed to the infection earlier than the controls that received no vaccine treatment. This seems to indicate that the resistance of the animals is lowered by the antigen treatments. Bronfenbrenner and Straub, and Kettel have also noted this fact in their experiments.

The vaccine treatment was generally followed by suppurative lesions, in only a few instances healing over with the crust formation mentioned by Dryer. In no instance, however, was this formation followed by recovery. All of our animals succumbed to the infection.

For bibliography see the original article.

IMMUNIZATION OF A HORSE AGAINST BOTULINUS TOXIN FROM B. BOTULINUS (NEVIN. TYPE B. AND ORR TYPE A).

Edwin J. Banzhaf and Herman Gerber.

The potency of the different lots of toxin used during the course of immunization varied from 0.001 to 0.00005 of a cc. The horse's resistance to botulinus toxin being unknown, the immunization was started cautiously. The first injection was 1/10 of a guinea pig M.L.D. (subcutaneously) on 3-18-20. The injections were increased every second day 100 per cent. Temperature reactions were slight, usually less than one degree, the horse showed no other disturbances and at all times during the entire course of immunization ate his full allowance of rations. After the 12th injection the increase of the toxin dose was dropped to 50%; after five weeks of immunization a test bleeding was taken and potency found to be about 1 unit per cc. Injections were continued at a 25 per cent. rate of increase. The 5-10-20 six liters of blood were drawn from the horse, potency 5 units. The sixth bleeding on 8-26-20 showed a potency of 200 units per c.c. After the ninth bleeding the horse was very much emaciated. The toxin injections from Nevin type B. were discontinued and an attempt was made to immunize the horse against botulin toxin (type A, Orr). The first injections was one guinea pig M.L.D. and increased 100 percent every second day. Three weeks later the horse was found down in the stall and was bled to death.

On November 29th, 1920, immunization was started in another horse against botulinus toxin (type A, Orr). The method of immunization of the previous horse was followed with the exception that the first injection was one guinea pig M.L.D. instead of 1/10 of an M.L.D. On January 13th, 1921, the horse was bled, and it tested 175 units per c.c. 500,000 M.L.D.s was the last amount injected previous to bleeding.

The fourth bleeding was taken February 28th, 1921 and tested 250 units per c.c. It was found that the botulinus antitoxin could be refined and concentrated by the Banzhaf method.

ON THE CLAIM THAT SOME TYPHOID-PARATYPHOID STRAINS SURVIVE THE MILK PASTEURIZATION.

Published in The Journal of Infectious Diseases, 1921, Vol. XXIX, pp. 310-312

Charles Krumwiede and W. Carey Noble

The statement of Schorer and Rosenau[1] that the thermal death points of the pathogenic organisms that may render milk dangerous have been determined with precision in many laboratories and that an actual exposure to 60° C. for 20 minutes would suffice to kill such nonspore-bearing organisms as B. diphtheriae, B. tuberculosis and B. typhosus voiced the general conclusion of all who had worked on this subject. Although the practical application of these facts to the pasteurization of large volumes of milk was another problem, exposure to this temperature, for the time given, had been uniformly considered as adequate, and this aspect of the subject was looked on as closed.

Recently, however, Twiss[2] has reopened the subject and used bacteria of the typhoid-paratyphoid group as test organisms. Her contention is that, in actuality, the temperatures of pasteurization are inadequate; that the method of using small samples of the heated milk for the determination of the death of the bacterium present does not exclude the possibility of the survival of a few organisms. She asserts that if the whole test sample (100 c.c.) be incubated it will not infrequently be found that a few bacilli have survived exposure to 60° and even to 65° C. for 30 minutes.

The practical importance of the question immediately presented itself, and we began testing a series of cultures from different sources. Twenty-seven typhoid cultures recently isolated from carriers, 7 paratyphoid A cultures, 12 paratyphoid B. culutres and 4 enteritidis cultures were subjected to experiments, using the ordinary temperature of pasteurization.

Milk was sterilized in the autoclave, cooled and tested for sterility. It was then distributed in measured quantities in sterile containers. The containers were placed in wire baskets on a perforated tray in a water bath, and the temperature of the bath and the milk raised to 60° C. After this temperature was reached, suspensions of the cultures in salt solution were poured into the milk. Rubber stoppers were inserted, the bottles shaken vigorously and quickly returned to the water bath in which they were completely submerged. The temperature of 60° C. was held during the pasteurization period of 15 minutes. Rapid cooling was accomplished by plunging the samples into a bucket of cold water held under an open faucet. This method of pasteurization excluded the preliminary heating of the bacteria while the temperature of the milk was being raised. In every one of these tests we used a volume of 100 c.c. of sterilized milk infected with the cultural growth from two 24-hour agar slants suspended in salt solution.

The inoculated milk samples were then incubated for 48 hours at 37° C. to allow multiplication of any organisms which survived. After incubation, samples in duplicate, 0.5 c.c. and 0.1 c.c. were plated in agar and 2 broth tubes were each inoculated with 0.1 c.c. Russell's medium was also inoculated from the infected milk, as well as from the inoculated broth tubes.

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[1] Jour. Med. Research, 1912, 26, p. 127.

[2] Journ. Infect. Dis., 1920, 26, p. 165.

None of the micro-organism tested survived a pasteurization period thus limited closely to 15 minutes at 60° C.

These experiments confirmed our previous experiences, as well as those of others. Whether or not there might be more resistant members of the typhoid-paratyphoid group could not be excluded as a possibility, although this seemed improbable. To exclude the possibility that Twiss was working with unusually heat resistant strains, we obtained some of the strains which in her experiments seemed to survive the usually accepted pasteurization period.

The resistance of these strains was tested in several ways. One set of experiments with 100 c.c. of milk was carried out as described, and no growth was obtained. Then, larger volumes were inoculated very heavily by using for each bottle the growth from 10 agar slants. Whether the milk was inoculated before or after reaching 60° C., the results were the same. No growth was obtained after 20 minutes or even after 10 minutes' heating at 60° C.

Repetitions of these tests, not recorded here, have given the same negative results.

It would seem that the apparent heat resistance of the strains reported by Twiss was due to the test method employed, namely, the use of cotton plugged flasks submerged to twice the depth of the milk. The possibilities of discordant results from this open method have been pointed out by Smith[3] and others.

CONCLUSIONS

There is no evidence that bacilli of the typhoid-paratyphoid group, even in small numbers, will survive heating to 60° C. for 20 minutes. To insure actual heating of all the sample and to exclude other sources of error in laboratory pasteurization tests, the sample container should be completely submerged in the bath.

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[3] Jour. Exper. Med., 1899, 4, p. 233.

A STUDY OF THE PRACTICAL VALUE OF THE FROST LITTLE PLATE METHOD IN THE ROUTINE COLONY COUNT OF MILK SAMPLES

Published in The American Journal of Public Health, 1922, Vol. XII, pp. 478-487

Hazel M. Hatfield and William H. Park

The method of growing bacteria in solid media on sterile slides was applied by W. D. Frost in 1915 to the routine bacteriological examination of milk. It was called by him the "little plate" method.

This method is a modification of the standard plate method for milk analysis in that the standard agar for milk work is used and the result obtained is a computation of the number of colonies per cubic centimeter of milk developing under conditions essentially those of the standard plate procedure. Since 1915, Frost, working at the University of Wisconsin, has repeatedly modified and simplified the technic and improved the necessary apparatus.

The present technic of the little plate method is not difficult. An assistant with good general laboratory training can learn to make the test without difficulty; workers with less general experience gain proficiency more slowly, but a few days of practice will usually suffice for accurate results.

The method[1] in brief is described by its author as follows:

Two areas of four square centimeters each are marked off with a wax pencil on an ordinary microscopic slide which has been carefully polished. The slide is sterilized by passing it back and forth through a Bunsen flame. It is then laid on the flat top of a copper box containing water at a temperature of 42° to 45° C. The milk sample is shaken gently to avoid the formation of foam, then 0.05 (1/20) c.c. of the milk is measured from a "serum pipette" (a narrow, accurately calibrated pipette measuring 0.2 c.c. graduated to 0.01 cc.) onto the ruled area at the left of the slide, and the same amount on the right end of the slide. An approximately equal amount of sterile nutrient liquified agar at 42° to 45° C. is added by allowing one drop to fall from a 1.0 c.c. pipette upon each measured drop of milk sample.

The milk and agar are thoroughly mixed with a sterile loop and carefully spread over the marked-off areas. This gives duplicate films. The preparation is set aside to solidify on a level surface under cover, and then, before the agar begins to dry down, the slide is placed in a moist sterile chamber. This moist cabinet is set in the incubator. The period of incubation should be long enough to allow the bacteria to grow into definite colonies, although they need not necessarily be visible to the naked eye. It is stated that four hours incubation at 37.5° C. allows the formation of good-sized colonies if the bacteria are actively growing in the milk at the time of testing. If the milk has come from cold storage, has been pasteurized, or is fresh and contains few bacteria, then seven or eight hours are necessary. If there is no particular hurry about the results, the plates may be left in the incubator from one day to the next, i.e., for fifteen or sixteen hours.

When the slides are removed from the moist chamber after incubation, they are dried immediately at a temperature of about 95° C. If only a few slides are to be dried, they may be laid on the surface of the above mentioned copper box, which will give a temperature of about 95° C. if the box is filled with boiling water. Where large numbers of slides are to be dried, an electric oven is necessary. This temperature dries the agar films quickly enough to prevent the film from cracking or peeling. The drying is accomplished in five minutes or less.

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[1] Frost, W. D., Improved Technic for the Micro or Little Plate Method of Counting Bacteria in Milk. Jour. Inf. Dis., XXVIII, 2, p. 176.

The slides are now ready for staining. They are first immersed for a minute or two in 95 per cent. alcohol containing 10 per cent. of glacial acetic acid to prevent the agar background from taking a deep stain. The acid alcohol is drained off and the slides are then put into the stain. This may be either Loeffler's methlylene blue diluted with three times its volume of distilled water, or thionine blue made up with carbolic acid and distilled water with the addition of acetic acid. The stain will act sufficiently in about two minutes, but no harm will result from a longer exposure. The slides should be thoroughly washed in tap water, then dried at about 95° C. on a hot plate or in an electric oven as described above.

The counting is done under a compound microscope. If the colonies are small or very numerous the higher powers must be used; if the colonies are larger and fewer the low power may be used. A number of fields are counted and an average count per field obtained. In order to express the result of the count in terms of "colonies per c c." of milk, it is necessary to know the area of the microscopic field. To determine this, the diameter of the field is read off on a stage micrometer, the radius is obtained by dividing the diameter by two and the following geometrical formula applied:

The area of a circle=radius squared × 3.14159. Then divide the area of the microscopic field into the total area of the film (4 square centimeters) and multiply the result by 20 because only 0.05 c.c. of milk is used to make the test. The result is a constant by which the average count per field must be multiplied in order to convert it into "colonies per c.c." of milk. This multiplier is a constant for each combination of lenses so tested but must be determined again for each microscope and for each new combination of lenses.

Heavily contaminated milk samples require dilution before this test can be carried out. The dilution advised is 1.0 c.c. of the milk to be tested in 9.0 c.c. of sterile milk. After thorough mixing, the diluted sample is used in exactly the same way as the ordinary sample. Another way, simpler than this, is also recommended: 0.01 c.c. or 0.02 c.c. of the undiluted milk sample is pipetted onto the ruled slide, a drop of warm sterile milk and the usual drop of agar added,—the whole mixed and spread in the usual way.

The advantages claimed by Frost for the little plate method are:

1. The time required to set up the little plate test is less than that needed for the standard plate test.
2. The incubation period is shortened so that routine results may be obtained in twenty-four hours and, in special cases, results may be obtained the same day.
3. The technic of the little plate method is simpler than the technic of the standard plate method.
4. The little plate method requires less material in the way of glassware and media. There is a corresponding saving of labor and expense in preparing these supplies.
5. It permits a study of a relatively large amount of milk, usually 0.05 c.c.

6. The slides are permanent preparations and can be filed for record and re-examined whenever the need arises.

7. The method furnishes direct information about the types of organisms present, their relative numbers, etc.

8. It is a method easily adapted to field work.

To determine how far the Frost little plate method is applicable to the work of a large routine milk laboratory run for administrative purposes, we have carried out an extensive series of tests on all grades of milk, controlling the test in all cases by standard plate counts. Whenever it was possible to do so, the standard plate was made in duplicate. A few minor changes have been made in the Frost technic where such modifications would expedite the work or make the test more like the standard plate method.

Our most important modification of Frost's technic concerns the shaking of the milk sample. Frost tips the sample bottle from end to end slowly enough to avoid the formation of foam, since air bubbles drawn into the pipette would interfere with the accuracy of measurement. For routine work, the manner of shaking milk samples is minutely prescribed by standard methods; it is a vigorous shake and will break up clumps more than the shaking recommended by Frost. In order to make a comparative study, with the standard plate results as the basis for comparison, it seemed essential to shake the milk sample in the same manner for both tests. In our laboratory, therefore, the sample is shaken in the manner prescribed by standard methods and then both tests are immediately carried out. Tipping the bottle after shaking causes the foam to float to one side and the milk may be drawn into the pipette without including air bubbles, thus overcoming the objection mentioned by Frost.

To save the time consumed in ruling the slides, a Wolf-Hügel counting plate is placed upon the warm box and the sterile slides placed upon the counting plate. The ruled lines of the counting plate are clearly seen through the slide and the milk-agar mixture is carefully spread over four square centimeters of the slide as defined by the lines beneath. Although we lose the advantage of having the mixture flow back from a greased line, we accomplish a slight saving in time on each test and this saving becomes considerable as the number of tests increases.

After staining the film, we prefer to decolorize slightly. This technical point was mentioned by Frost in his earlier articles, but is not mentioned in his most recent article. The count seems to be uninfluenced by decolorization if this is not carried too far. A few seconds in alcohol are all that should be allowed. Films that have been overdecolorized may easily give too low a count, as unstained colonies cannot always be picked out with accuracy.

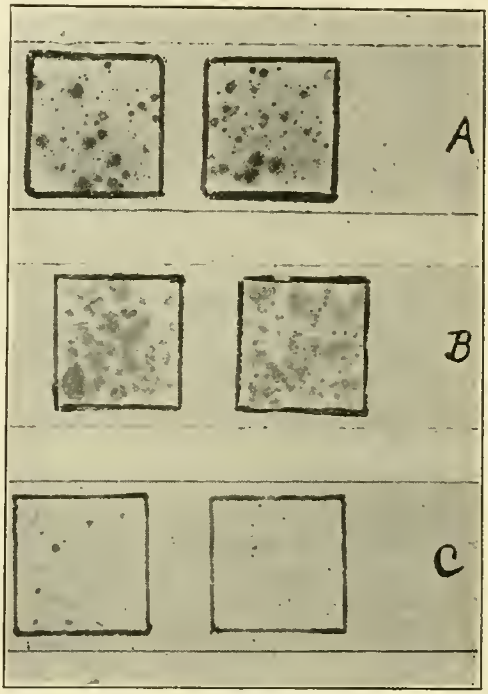
The accompanying photograph shows the stained preparation ready for counting.

In all the work used as the basis for this article, only one time-period for incubation has been employed. The moist chamber containing the slide preparations was put into the incubator at 5 p. m. and was taken out at about 8 a. m., thus making a fifteen to sixteen hours incubation. As pointed out by Frost, it is desirable to incubate over night when dealing with the test in a routine way, otherwise it is necessary to be at the laboratory out of hours. With a large volume of routine work, the overnight incubation is important to the indorsement of the method. Although Frost's work is based upon an incubation period of four to eight hours, the overnight incubation is equally recommended by him. It is probable that any difference in results is rather in the size of the colony than in the count.

When we began this work, colony counts were made in an absolutely mechanical way by counting five or ten fields of each film, going diagonally across the film in both directions and averaging these results. As the work went on, however, and our experience grew, we found that with ordinary care in making the films, the colonies were so evenly distributed that equally accurate results could be obtained by counting two or three representative fields from each film. It is preferable to do this with a mechanical stage.

Plate Sheet

The appearance of the little plates after staining is shown in the accompanying photograph.



Little plates in duplicate on slides after staining (fifteen hours incubation). *A*—Moderate number of colonies; *B*—High count, more uniform colonies; *C*—Small number of colonies. (Reprinted by courtesy of The American Journal of Public Health.)

Some of the assistants in our laboratory have also been tried out on the counting. They have required little practice before obtaining counts very like our own. It must be borne in mind, however, that these girls have had years of experience in counting milk plates. Their opinion is that the stained little plate preparation is easier to count and entails less eye-strain than does the standard milk plate.

As a rule, the count is not radically affected by the choice of an objective. (See Table 1.) Of course, where the count is very low, the oil immersion is impracticable because many fields will show no colonies, hence it is difficult to get a true average count per field, and the factor for multiplication is so large that a slight variation in the average count is multiplied enormously.

TABLE 1.
COMPARATIVE COUNTS ON MILK SAMPLES WITH SPECIAL REFERENCE TO THE USE OF VARIOUS OBJECTIVES.

	COLONIES PER C.C. OF MILK					
	Frost Little Plate Method				Standard Plate Method	
Sample No.	Amt. Tested	No. 3 Objective	No. 6 Objective	Oil Immersion	Dilution Used	Count per c.c.
1	.05 c.c. .01 c.c.	Overcrowded 2,741,892	 2,624,058	2,360,000 2,000,000	.... 1 1,000	 2,500,000
2	.05 c.c. .01 c.c.	37,996 *	60,416 *	90,000 *	1:1,000	65,000
3	.05 c.c. .01 c.c.	172,983 114,433	183,330 187,497	220,000 too few	1:1,000 1:10,000	224,000 210,000
4	.05 c.c. .01 c.c.	34,330 *	70,832 *	80,000 *	1:1,000	45,000
5	.05 c.c. .01 c.c.	Overcrowded 4,452,000+	1,894,279 4,977,989?	1,973,552 3,788,515	1:10,000	11,000,000
6	.05 c.c. .01 c.c.	595,720 1,297,440	572,689 678,416	722 461 704,840	1:10,000	1,200,000
7	.05 c.c. .01 c.c.	Many millions Many millions	Many millions Many millions	Many millions Many millions	1:100,000	223,200,000
8	.05 c.c. .01 c.c.	Overcrowded 11,872,000	4,390,000? 14,096,960?	Col. run together 7,224,610	1:100,000	27,000,000
9	.05 c.c. .01 c.c.	1,225 360 1,250,800	1,057 272 1,718,067	1,074,881 1,057,250	1:10,000	2,400,000
10	.05 c.c. .01 c.c.	Many millions Many millions	Many millions 10,572,720	Col. run together 9,162,920	1:10,000	124,620,000
11	.05 c.c. .01 c.c.	Overcrowded 3,697 280	1,392,075 2,731,283	1,797,342 2,746,655	1:1,000	3,200,000
12	.05 c.c. .01 c.c.	529,900 712,320	405,288 616,742	334,799 792,945	1:1,000	776,000
13	.05 c.c. .01 c.c.	Many millions Many millions	Many millions Many millions	Many millions 32,775,060+	1:10,000	137,640,000
14	.05 c.c. .01 c.c.	3,595,520+ 6,784,000+	4,365,330? 5,286,360?	2,625 529 Colonies indistinct	1:10,000	10,800,000
15	.05 c.c. .01 c.c.	850,120 1,036,680	740,090 660,795	599,114 Growth too diffuse	1:10,000	1,100,000
16	.05 c.c. .01 c.c.	295,104? 560,740	208,376 *186,050	881,050? Diffuse growth *176,210?	1:100 1:1,000 1:10,000	273,000 400,000 350,000
17	.05 c.c. .01 c.c.	62,208 58,300	54 573 *	* *	1:100 1:1,000 1:10,000	36,000 51,000 120,000
18	.05 c.c. .01 c.c.	24,168 27,560	24,807 *	* *	1:100 1:1,000 1:10,000	24,000 28,000 100,000
19	.05 c.c. .01 c.c.	366,316 312,640	282,796 *	328,925 *	1:100 1:1,000 1:10,000	227,500 584,000 600,000
20	.05 c.c. .01 c.c.	35,616 46,640	* *	* *	1:100 1:1,000 1:10,000	28,800 53,000 60,000

Samples Nos. 3, 5, 7, 10, 13, 16, 19 were diluted 1:10 to makes samples Nos. 4, 6, 8, 11, 14, 17, 20, and these in turn were diluted 1:10 to make samples Nos. 9, 12, 15 and 18.

NOTE: These milks contained an excessive number of lactic acid bacteria, having been incubated at room temperature over night.

*Too few for accurate count.

If the milk has a very high bacteria content, the field under the low power is too overcrowded to be accurately counted and the oil immersion is much more satisfactory. In the latter case the count may be too low even with the oil immersion because of the inhibition of colony development due to overcrowding, hence the testing of a smaller amount of milk is indicated.

In either case, the important fact for grading purposes is easily ascertained—a very high or a very low count is quickly recognized and a medium count can be made with any objective. Occasionally a milk is found which gives a higher count when the magnification is increased. This may be due to a different type of milk or to artifacts. (See Table 2.) These cases require further study. In this connection, it must be borne in mind that the oil immersion shows the individual bacteria and that clumps of these cannot, at times, be differentiated from beginning colonies. The possible effect of an unusual flora is shown by the following case:

Thirty-nine samples of pasteurized milk, all from one source, were run in parallel by the two methods. The standard plates gave counts ranging from 62,000 to 710,000 per c.c. and showed apparently an almost pure culture of an organism producing extremely small colonies. The Frost plates all gave counts under 10,000 per c.c. when examined with the No. 3 objective. With the No. 6 objective the counts were somewhat higher. With the oil immersion lens very few colonies could be found, but many fields showed a mass of organisms numbering many hundreds, all of the same general appearance, but no denser or more deeply stained in one part of the mass than in another.

Table 2 gives results on five of these samples picked at random.

TABLE 2.

EFFECT OF PRESENCE OF UNUSUAL FLORA

Little Plate Counts Increased with Increased Magnification. Standard Plate Counts Still Higher

		Frost Little Plate Count		
Sample No.	Standard Plate Count	No. 3 Objective	No. 6 Objective	Oil Immersion Objective
894	91,000	6,500	33,333	Diffuse growth instead of colony formation. No Colonies seen.
4062	92,000	6,000	37,499	
7042	62,000	5,333	41,666	
7456	110,000	10,322*	41,666	
599	192,000	2,333	41,666**	

*Possibly much higher count.

**Very indistinct.

In general, the lowest possible magnification consistent with accuracy should be used, because it requires the smallest factors for multiplication and because the oil immersion introduces the danger of including the above-mentioned clumps in the colony count.

The making of proper dilutions in a rapid manner is a matter of great importance. It is true equally for the standard plate and for the little plate that the amount of milk tested should depend upon the probable bacterial content of each sample as indicated by the grade designated on the label. Where the counts are actually far in excess of this, conditions are not suitable for the development of all potential colonies and the count per c.c. is less than it would have been from a higher dilution.

For the better grades of milk, the samples were tested undiluted, using

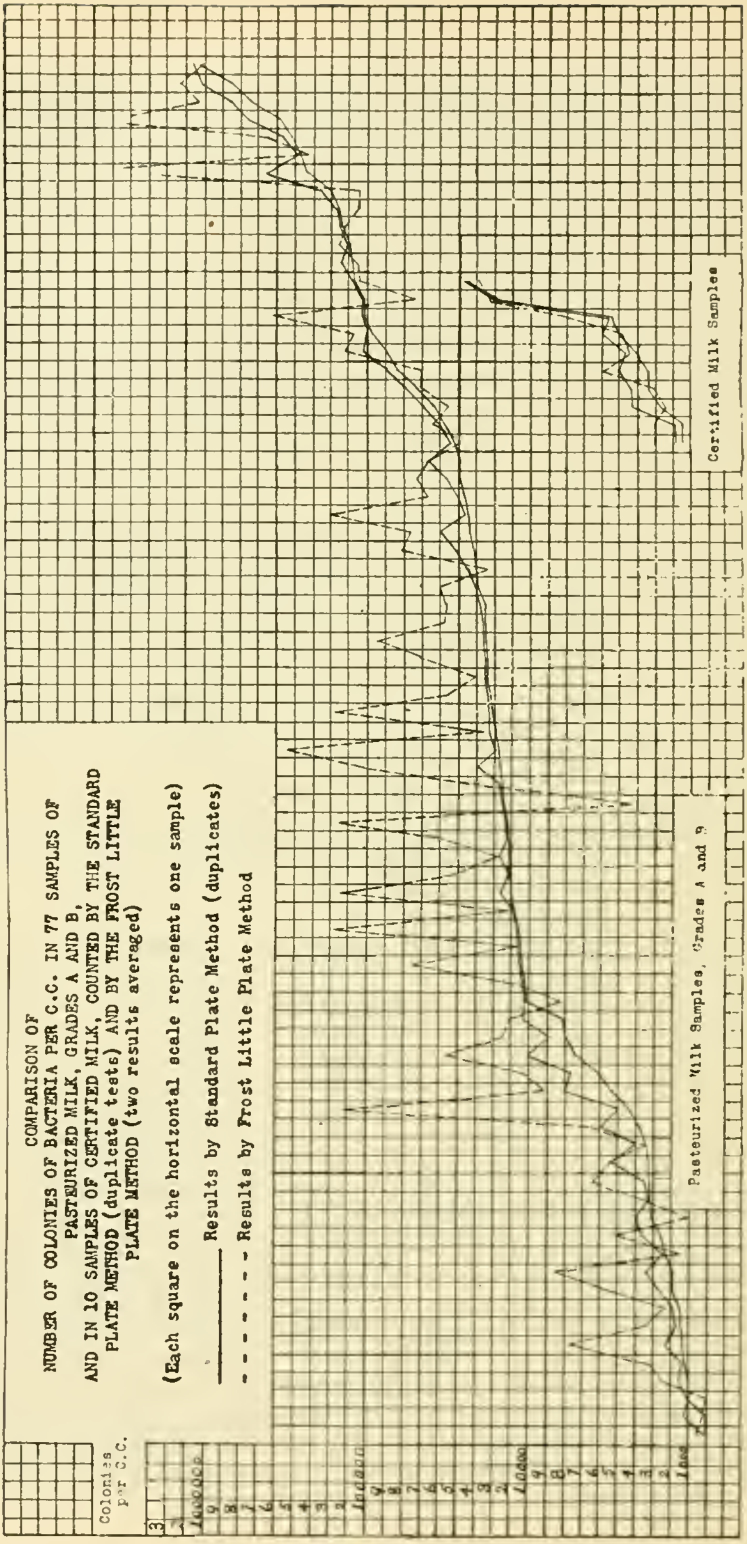
0.05 c.c. of milk and about an equal amount of agar. For the more highly contaminated milks various ways of examining a smaller portion of milk were tried, in order to give the potential colonies room to develop. This, of course, meant a higher factor to be used as a multiplier for the average field. At first, the samples were diluted 1:100, using sterile milk as a diluent; of this dilution, 0.05 c.c. was plated. This technic was unsatisfactory because there were too few colonies in the film.

Next, the milk sample was similarly diluted 1:20 with sterile milk and the usual 0.05 c.c. plated. This method was more satisfactory than the dilution of 1:100, yet often even the 1:20 dilution gave films showing too few colonies. In any case, the making of a dilution was time-consuming and we wished to do the work more directly if possible. To this end we tried the examination of 0.01 c.c. of undiluted milk, adding to it 0.04 c.c. of sterile milk and the usual drop of agar. This procedure was time consuming also and the 0.01 c.c. of milk begins to dry down before the sterile milk can be accurately measured and added.

The method eventually adopted by us for the testing of highly seeded milk samples consists in the examination of two different quantities of the undiluted milk sample, 0.05 c.c. on the left end of the slide and 0.01 c.c. on the right end of the slide without the addition of sterile milk. This has the advantage of simplified technic and the elimination of a sterile diluent with its attendant work of preparation and possibility of contamination. The lessened milk content of one of the finished preparations does not seem to make as much difference in the count as we anticipated. Although it is easily conceivable that milks containing large numbers of certain types of lactic acid bacteria would fail to show a corresponding colony count in the film containing less milk, yet the same objection is true of the standard plate which actually contains even less milk. As this is a study based on comparisons with the standard plate results, we believe that the above procedure is admissible. Moreover, the finding of a reasonable colony count ratio between two films containing different amounts of the same milk sample is reassuring. It is comparable to the colony count ratio between two or more standard plates containing different quantities of a given milk sample. In each case, due allowance is made for inhibition due to overcrowding. For field work, the elimination of a sterile diluent also means fewer supplies to carry about, a less complicated procedure in an improvised workroom and a shorter time required for setting up the test.

The results of our work, on the whole, have been more clear-cut than we had anticipated. The certified milks showed the smallest average variation from the standard plate result. The pasteurized milks and raw milks to be pasteurized gave greater variations, these being divided about equally between results greater than standard plate counts and results less than standard plate count and ranging from —28.6 per cent. to +18.7 per cent. Table 3 presents an analysis of the counts obtained from 241 samples of milk tested by both methods. Only later results are tabulated here because they represent a more experienced handling of the little plate method and the use of 0.05 c.c. and 0.01 c.c. of undiluted samples of raw milk.

These percentages are somewhat higher than those obtained from duplicate standard plates made during the course of this study. The duplicate standard plates gave an average variation of 9 per cent. on 87 samples of certified and pasteurized milk. These duplicate standard plate counts with the corresponding Frost little plate counts are graphcially shown in the accompanying Chart 1.



(Reprinted by courtesy of The American Journal of Public Health.)

CHART 1.

TABLE 3.

CORRESPONDING AVERAGE COUNTS BY THE STANDARD PLATE METHOD AND THE FROST "LITTLE PLATE" METHOD.

Grade of Milk	Legal Maximum Colony Count per c.c.	Number of Samples Included	Average Colony Count per c.c.		Little Plate Variation from Standard Plate
			Standard Plate	Little Plate	
Certified	10,000	22	4,484	4,330	-3.5%
		(24)*	(14,660)*	(16,468)*	(12.4%)*
A. Pasteurized.........	30,000	15	15,220	18,067	18.7%
B. Pasteurized.........	100,000	47	83,874	98,757	17.7%
A. Raw..............	200,000	66	73,403	84,921	15.7%
**B. Raw (country)...	300,000	48	1,377,917	984,182	**-28.6%
B. Raw (city)	1,500,000	41	693,793	591,101	-14.8%

*These entries represent the same 22 samples of certified milk shown above plus 2 very high count samples (see Table 2). It can be seen how quickly the average variation by the two methods is altered when excessively high count samples are included.

**More than half of these samples were 48 hours old and the counts on many were too high for exact findings with the present way of making dilutions for the little plate test.

In Table 4 comparative results by the two methods are given in detail for each grade of milk. These results have been selected from many hundreds of similar results in such way as to convey a fair idea of the usual agreement by the two methods and the degree of occasional disagreement.

It will be seen (Table 4) how rarely the comparative counts fail to coincide in rating the milk above or below standard. In not more than 2.0 per cent. of all samples tested would the legal rating have been altered by the method of counting; the milks were either above or below standard by both methods. The exceptions noted were about evenly divided between those cases where only the standard plate gave a count in excess of grade and the cases where only the Frost little plate gave a count in excess of grade.

Summary.—In the hands of properly trained workers the Frost little plate count is probably as accurate and reliable as the standard plate count for routine laboratory work, especially in the case of pasteurized, certified and Grade A raw milks with the rare exception of milks containing large numbers of certain unusual types of bacteria. Where the counts exceed a million, as they often do for Grade B raw milk (the legal limit for which is 1,500,000 per c.c.), a greater discrepancy between the two methods sometimes appears. Even here the rating is seldom affected by the method used, but it is evident that the dilution technic for the Frost little plates must be further modified to take care of very highly contaminated milks and those containing unusual types of bacteria.

TABLE 4.

Grade of Milk	Legal Maximum per c.c.	Colonies per c.c.	
		Standard Plate	Frost Little Plate
Certified	10,000	6,660	8,100
		4,400	4,300
		3,600	2,600
		4,600	3,100
		5,000	5,900
		6,500	4,800
		1,800	3,100
		1,800	3,100
		*141,200	*147,300
		*112,000	*152,600
		1,600	2,000
		1,800	3,000
		900	300
		1,600	1,600
		*12,000	8,800
		*16,500	*18,900
		3,000	2,500
		1,100	4,000
		3,500	2,000
		3,600	1,300
		6,700	7,100
		10,000	6,600
		2,200	1,100
		2,200	1,000
A. Pasteurized	30,000	*Plate overcrowded	*2,446,400
		4,600	18,900
		12,000	10,900
		9,000	13,900
		3,500	4,800
		2,000	3,300
		*80,000	*93,300
		20,000	19,400
		*38,000	*37,000
		6,200	9,000
		6,000	5,000
		5,000	5,000
		*61,700	*76,600
		13,600	28,900
		3,300	3,000
		7,500	6,000
		8,000	5,000
		2,300	4,000
		*400,000	*144,000
		5,300	6,000
		18,600	24,200
		30,000	*37,900
B. Pasteurized	100,000	28,000	26,100
		43,000	85,800
		3,900	7,000
B. Pasteurized (Continued)	100,000	*147,000	*133,000
		*182,000	*319,900
		*223,000	*273,000
		88,000	50,600
		41,000	33,300
		80,000	100,000
		44,000	37,200
		*170,000	*159,000
		*120,000	86,700
		24,000	24,000
		*640,000	*773,300
		61,500	70,000
		20,000	27,000
		6,600	8,000
		3,900	7,000
		59,000	44,900
		65,000	26,500
		96,000	19,600
		*260,000	*139,200
		*420,000	*599,900
		43,000	64,900
		*110,000	*129,900
A. Raw	200,000	36,000	35,800
		42,000	46,600
		58,000	53,300
		104,000	123,300
		120,000	138,300
		144,000	199,900
		80,500	94,900
		110,000	84,900
		100,000	121,600
		96,000	120,600
B. Raw ..... Collected in Country	300,000	240,000	296,600
		92,000	92,000
		*1,800,000	*1,866,400
		*2,300,000	*2,899,700
		250,000	283,300
		*556,000	*783,300
		*556,000	*599,900
		*500,000	*366,600
B. Raw ..... Collected in City	1,500,000	160,000	186,500
		630,000	491,600
		140,000	199,900
		42,600	49,900
		660,000	473,200
		*1,650,000	*1,606,600
		240,000	233,300
		*2,650,000	*1,733,100
		*2,200,000	*1,666,500
		155,000	166,600
		940,000	1,116,500

NOTE: Stars indicate counts in excess of legal maximum.

Conclusions.—Our present opinion is that both methods are to be depended upon and that, according to the local conditions, one or the other may be utilized.

Whether this test supplants the standard plate method or not, it is a matter of satisfaction to have a second test that can be run in parallel whenever check tests are made.

For bibliography see the original article.

STANDARDS FOR MILK PASTEURIZATION

Published in The American Journal of Hygiene, 1927, Vol. VII, pp. 147-173

By Charles E. North and William H. Park

There has just been reported the occurrence of an outbreak of septic sore throat in Guilford and adjoining towns in Connecticut. During July and August there were 200 cases. Eighty per cent. of those affected were consumers of milk from a single dairy. This milk was raw and unheated. On the farm was a milker convalescing from septic sore throat, through whom some of the cow's udders were infected and from these the milk infection started.

This is only another one of many such instances. Epidemics of typhoid, scarlet fever, septic sore throat, and diphtheria have been so often traced to raw milk that pasteurization of public milk supplies is recognized as a public necessity. The constant menace of bovine tuberculosis adds its weight to this demand. The infections of infants and children by raw milk and the benefits of pasteurized milk for infant feeding are now part of medical knowledge.

But the term "pasteurization" itself still has an indefinite meaning. The temperatures and times originally used by Pasteur for wines and beers are not sufficient for milk. The use of the process for milk, first for the protection of calves and pigs, later on for the protection of infants and children, and finally for the protection of the general public, has resulted in the adoption of different legal requirements by many cities and states. A survey of the legal standards, however, reveals a marked disagreement both as to the temperature of heating and the time of exposure. Many local health authorities have adopted local requirements without an accepted principle as a basis, and without recognizing the definitions used elsewhere. There are at present no uniform standards accepted by health authorities in the United States. As a consequence public confidence in the process of pasteurization and in pasteurized milk has suffered. The lack of such standards is therefore detrimental to public health.

The bovine tubercle bacillus is more resistant to heat than any of the other species of pathogenic bacteria commonly carried by milk. Therefore, standards for pasteurization must be based on standards for the destruction by heat of the bovine tubercle bacillus.

In the classical experiments of Theobald Smith there were 83 samples of milk pasteurized at 140° F. for various periods of time. From these. 83 guinea pigs were inoculated. From the samples heated for shorter periods, the pigs gave positive results. The longer periods gave negative results. Smith's opinion that these data were enough to justify the conclusion that tubercle bacilli are killed at 60° C. (140° F.) in 20 minutes has been universally accepted by bacteriologists.

Russell and Hastings in 1900 tested 30 samples on 60 guinea pigs in one experiment and 18 samples on 36 guinea pigs in another. These data were deemed sufficient to warrant the conclusion by them that tubercle bacilli are killed at 60° C. for 20 minutes.

Rosenau in 1908 tested 87 samples of milk with 87 guinea pigs, and the evidence was sufficient in his opinion to support the conclusion that tubercle bacilli are killed at 60° C. for 20 minutes.

The group of bacteriologists working at Endicott in 1922-23 performed the following tests, which are reported in Bulletin No. 147 of the U. S. Public Health Service:

Temperatures	Number of Samples	Number of Guinea Pigs	Table Nos.
135° F. to 137° F.	27	54	19
130 " 140	27	54	20
134 " 145	122	244	34
141 " 142	19	38	35
130 " 147	90	180	36
142 " 150	64	128	67, 71, 75, 79
138 " 142	92	184	68, 72, 76, 80, 89
138 " 146	106	212	69, 73, 77, 81, 85, 90
138 " 148	171	342	70, 74, 78, 82, 86, 95

These tests show totals of 718 samples tested on 1,436 guinea pigs at from 130° F. to 150° F. This is the volume of data on which Moore, Park, Rosenau, Wadsworth, Armstrong and North base the statement that the tubercle bacillus is killed at 138° F. in 30 minutes, and that 142° F. for 30 minutes is a standard for pasteurization that provides a margin of safety sufficient to protect the public health against milk-borne infections. The data in these tests are more abundant than the data in the tests of Smith, Russell and Hastings, and Rosenau, which no one has yet questioned.

Bulletin No. 147 of the U. S. Public Health Service concludes that the tubercle bacillus is killed at 138° F. for 30 minutes. This is some help in extending the curve below the classical point of 60° C. for 20 minutes. But there still remains some uncertainty regarding the effect of low temperatures on the tubercle bacillus, because no systematic research on the various temperatures below 60° C. has yet been performed.

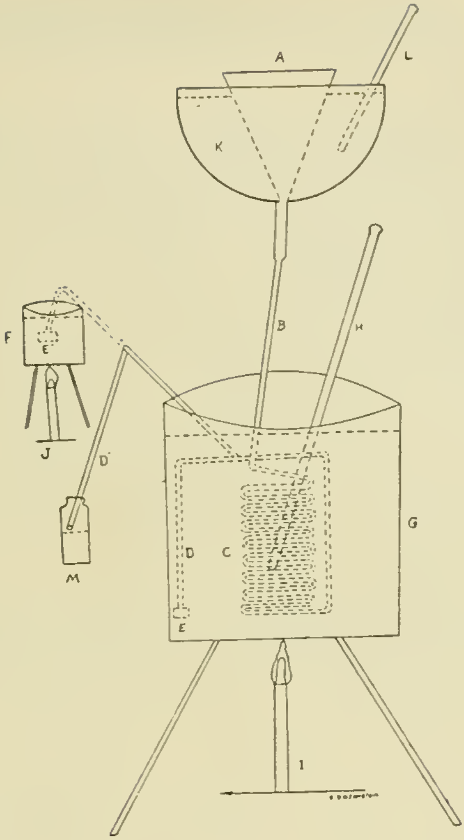
The experiments reported herein were undertaken to supply this deficiency.

It cannot be claimed that all sources of error of previous investigators were eliminated. But plans were made to prevent a repetition of the errors previously enumerated. It is true that standard conditions for the heating and testing of the tubercle bacillus are not yet recognized. Good management and good planning are as important as technical skill in making research work successful. The conditions planned and used in this research were as follows:

Culture. The culture used was of the bovine species known as the "Ravenel" culture, furnished by Dr. M. P. Ravenel to Dr. Wm. H. Park several years ago. This had been kept alive on glycerine agar and was cultivated on glycerine broth until abundant growth was obtained.

Virulence. One one-thousandth of a milligram injected subcutaneously produced in a guinea pig a tubercular abscess at the point of inoculation, also tubercles in the superficial axillary and inguinal nodes, in the lungs, kidneys, and spleen at autopsy, 49 days after inoculation. Also, one one-thousandth of a milligram injected intravenously in a rabbit produced extensive tubercles of the lungs and kidneys and isolated tubercles in the spleen.

Mixture. The growth on the surface of the glycerine broth was ground in a mortar with sterile salt solution until a smooth uniform suspension, so



Heating equipment for testing thermal death points of bovine tubercle bacillus.

A......Funnel supplying tuberculous milk
B......Rubber tubing from funnel to lead coil
C......Lead coil
D......Rubber tubing outlet from lead coil
E......Metal plug at end of outlet
E'.....Metal plug in sterilizing tank
F......Sterilizing tank
G......Water tank for heating lead coil
H......Thermometer
I......Burner
J......Burner
K......Water bath for funnel
L......Thermometer
M......Sample bottle receiving heated milk

(Reprinted by courtesy of American Journal of Hygiene.)

far as possible, was obtained. This was filtered through coarse filter paper to remove any accidental lumps. This suspension was then mixed with sterilized milk, the quantity of tubercle bacilli in each cubic centimeter being about twenty times the quantity already found necessary to kill a guinea pig by the virulence tests.

Equipment. Full consideration was given to all of the varieties of heating equipment used by previous investigators. The primary object of the process was to heat tubercle bacilli uniformly within a fraction of one degree of any chosen temperature for any chosen period of time. The type of apparatus used by MacFadyen and Hewlett in 1897 seemed the most certain to accomplish these purposes. This in principle consists of a coil of small calibre lead pipe, submerged in a hot water bath. At each end of the lead pipe are attached pieces of rubber tubing, one for inlet and the other for outlet. The inlet rubber tube receives the supply of tuberculous milk from a glass funnel kept warm by a warm water jacket. The outlet rubber tube is stoppered with a metal plug which is removable. The lead coil is heated by the water in which it is submerged. The water is heated in a metallic pail or tank to any degree desired, as indicated by a thermometer suspended in the water. There is a small secondary vessel of boiling water to be used for sterilizing the end and metal plug of the outlet pipe. This method of heating was used for all temperatures under 180°. In the three highest temperatures, open tubes over a flame were utilized, and, except for the boiling temperature, are not strictly accurate.

Operation. To operate this heating unit the glass funnel is filled with milk and this is warmed to the desired temperature, and the hot water surrounding it is heated two degrees hotter. The emulsion of tubercle bacilli in milk is heated to about 100° F. The water in the main tank containing the lead coil is previously heated to the desired degree for a length of time sufficient to make certain that the lead coil is thoroughly heated. The rubber tubing at the outlet end, with its metal plug, is submerged under the water and also heated. When the time in minutes and seconds for the beginning of the test is noted and recorded, the milk emulsion of tubercle bacilli is added to the heated milk, the mixture is vigorously stirred for 3 seconds and and allowed to run into the lead coil. To do this the rubber tubing outlet is lifted out of water with forceps, the metal plug removed and held open until the tuberculous milk has flowed by gravity through the lead coil and appears at the outlet. The whole operation takes about 5 seconds. About 10 c.c. of overflow is caught in a sterile test tube and the metal plug replaced. The plug and the end of the rubber tubing is then sterilized by dipping in boiling water for a second and at once is submerged in the water of the main tank. In this way, at intervals of time selected for the experiment, small portions of about 10 c.c. of the tuberculous milk can be withdrawn into a series of test tubes which are immediately placed in a cold water bath and cooled.

The advantages of this method of heating are worthy of special comment in view of the great irregularities of the results due to imperfect heating equipment. When the lead coil has been filled with tuberculous milk, it is closed at both ends. At the inlet end it is closed by surplus milk in the inlet pipe. At the outlet it is closed by the metal plug. The complete filling of the coil removes all air. The milk in the coil, therefore, is not in contact with outside air. As a result, the formation of pellicle, foam or splash is impossible. The submerged coil is uniformly heated, as the hot water in the bath is stirred. Therefore the milk in the coil is uniformly heated. By the movement of the milk through the coil it is also thoroughly mixed. The milk, which first fills the coil, remains at the temperature of the coil until it is withdrawn at the outlet. Therefore the period between the filling of the coil and the withdrawal of any portion of milk is the length of time the milk was heated. The amount of milk in the coil at the first filling is sufficient for all of the portions needed for a single test series.

Testing the milk. The milk was tested by the inoculation of guinea pigs. About 10 c.c. of milk was the portion withdrawn in each of the series of test tubes representing the different periods of time. These tubes, after cooling, were centrifuged for five minutes, and two guinea pigs were inoculated with one c.c. of the liquid from the top and from the bottom of each tube.

Thus the cream and the sediment of the milk in each tube was tested. The guinea pigs were kept in suitable cages for ten weeks and were then killed and examined for tubercular lesions. Any pigs dying before the expiration of that time were examined at death. Where typical tubercular lesions were found, the results were reported as positive. Where no tubercular lesions were found, they were reported as negative. Where the lesions were of doubtful character or suspicious, they were macerated in sterile salt solution and subinoculations made in additional guinea pigs.

Test series. A total of fifteen separate series of tests were made. The first three were in open vessels; the remaining 12 in the lead coil. The water tank was heated to twelve different temperatures at different times. These temperatures were: 170°, 160°, 155°, 150°, 145°, 142°, 140°, 138°, 136°, 134°, 132°, Fahrenheit. From the records of previous investigators, the approximate limits of time within which the tubercle bacillus would be killed at each temperature were known in advance. Therefore the ranges of times chosen for each temperature were selected with this information as a guide.

TABLE 1.
THERMAL DEATH CURVE OF TUBERCLE BACILLUS.

Temperature.	10 sec.	20 sec.	30 sec.	45 sec.	1 min.	2 min.	2½ min.	3 min.	4 min.	5 min.	6 min.	7½ min.	8 min.	10 min.	12 min.	12½ min.	15 min.	17½ min.	20 min.	22½ min.	25 min.	30 min.	35 min.	40 min.	45 min.	50 min.	60 min.	75 min.	90 min.
212°..............	— —	— —	— —																										
200°..............	.	— —	— —																										
180°..............	..	— —	— —																										
170°..............	..	— —	— —	— —	— —																								
160°..............	..	— —	— —	..	— —	— —																							
155°..............	..	..	— —	..	— —	— —	..	— —																					
150°..............	..	..	+ +	..	+ +	— —	..	— —	— —	— —																			
145°..............	..	..	+ +	..	+ +	+ +	..	..	+ —	..	— —	..	— —	— —	+* —														
142°..............	..	..	..	..	..	..	+ +	..	..	— —	..	— +	..	— —	..	— —	— —	— —	— —										
140°..............	..	..	..	..	..	..	..	..	....	+ +	..	..	..	— —	..	..	— —	— —	— —	— +*									
138°..............	..	..	..	..	..	..	..	..	..	+ +	..	..	..	+ —	..	..	+ +	..	— —	..	— —	— —	..	— —					
136°..............	..	..	..	..	..	..	..	..	..	..	..	..	..	+ —	..	..	+ —	..	+ +	..	+ +	— —	..	— —	..	— —			
134°..............	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	+ +	..	..	— +	..	— —	.. ..	— —	— —	..	..
132°..............	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	+ —	..	..	+ +	..	— —	— —	— —
130	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	+ +	..	..	..	..	— —	..	— —

*Explained as due to fresh material overflowing at end of operation.

RESULTS

The results of all of the autopsies on guinea pigs are shown in Table No. 1.

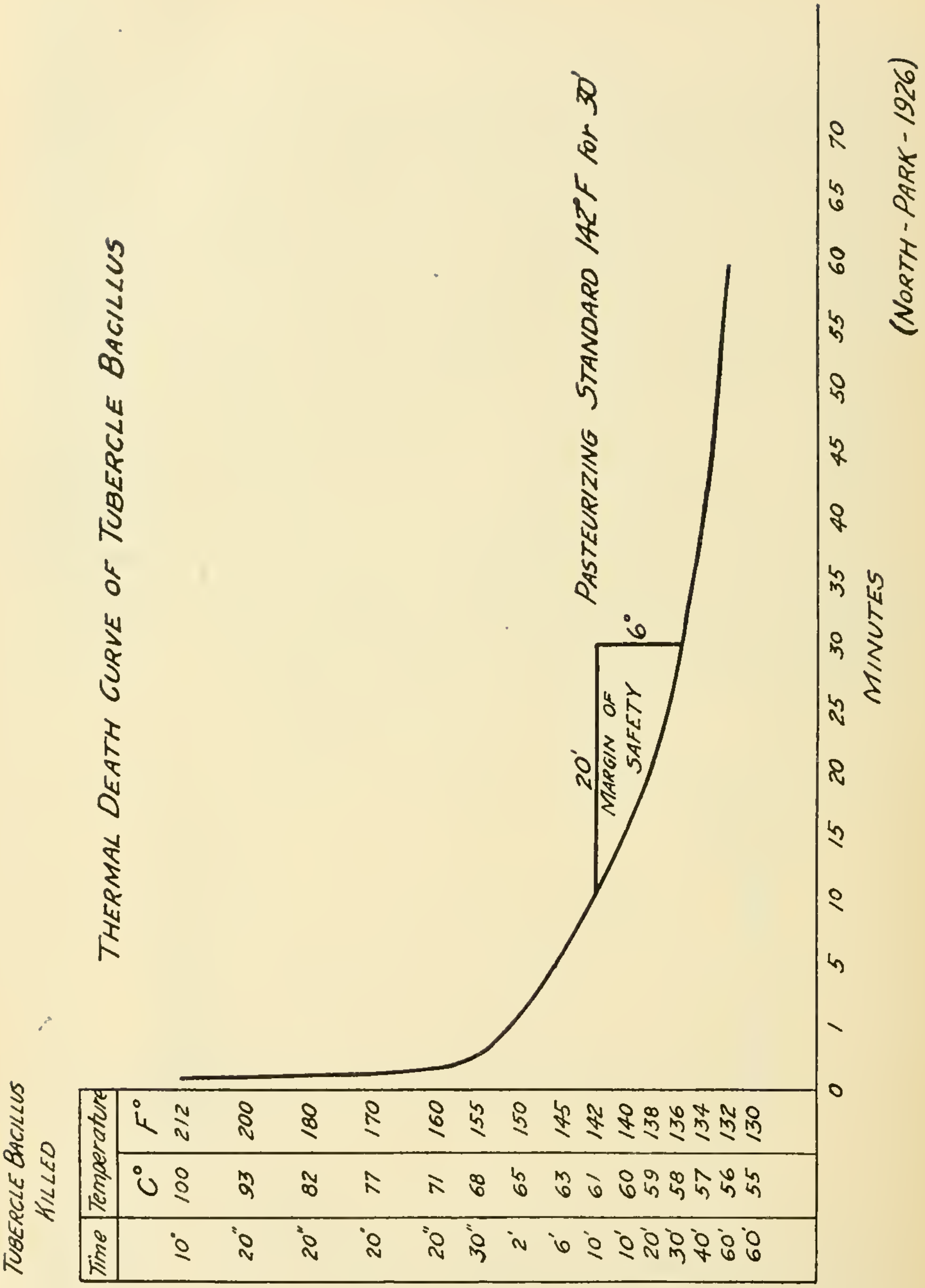
In this table the minus signs indicate the guinea pigs that were negative and the plus signs those that were positive. The results seem to be consistent in that the higher temperatures gave negative results in shorter times while the lower temperatures gave negative rsults at longer times, with the exception of the temperatures of 145° and 140°. In the case of 145° there were negative results in 6, 8, and 10 minutes, with 2 guinea pigs used at each temperature. On the other hand, at 12 minutes there was 1 positive and 1 negative result. This, however, was the last sample of milk taken from the apparatus, and it is believed that an error was committed by the operator in removing more milk than was contained in the coil at its first filling, so that fresh milk was allowed to come through which had not remained in the vessel the full period of time. In the case of the temperature of 140°, 2 negative results were obtained in 10, 15, 17½, and 20 minutes. This corresponds to the results obtained by Theobald Smith, Russell and Hastings and others at this temperature. On the other hand, at 22½ minutes one negative and one positive result occurred. This also was the last sample of milk taken from the apparatus, and as in the former case is believed to have been some fresh milk which had not remained in the coil a sufficient length of time. In this series of tests the results at other temperatures and also the results of previous investigations justify the conclusion that the positive guinea pigs from these two cases were due to errors by the laboratory worker.

If the temperatures and times at which negative results were obtained are arranged in order they give the following tabulation:

THERMAL DEATH POINT OF THE TUBERCLE BACILLUS

Temperature	Time
212° F	10 sec.
200	20 sec.
180	20 sec.
170	20 sec.
160	20 sec.
155	30 sec.
150	2 min.
145	6 min.
142	10 min.
140	10 min.
138	20 min.
136	30 min.
134	40 min.
132	60 min.
130	60 min.

If these points are arranged on a chart in which the intervals of temperature and time are spaced regularly and are connected with a line, such line forms a remarkably smooth curve, as shown in Chart 1. This curve may be properly designated by the title "Thermal death curve of the tubercle bacillus," since it contains within itself all of the infinite number of thermal death points of this species for any temperature and any time within the limits used.



TUBERCLE BACILLUS KILLED

Time	Temperature C°	Temperature F°
10'	100	212
20"	93	200
20"	82	180
20'	77	170
20"	71	160
30"	68	155
2'	65	150
6'	63	145
10'	61	142
10'	60	140
20'	59	138
30'	58	136
40'	57	134
60'	56	132
60'	55	130

CHART No. 1

(Reprinted by courtesy of American Journal of Hygiene.)

MARGIN OF SAFETY

The chief importance to public health of an accurate determination to establish the thermal death curve of the tubercle bacillus, such as has been attempted herein, is to lay the foundation for standards for the pasteurization of milk. It is obvious that no standards for pasteurization should be made identical with any of the temperatures and times necessary to kill the tubercle bacillus, since this would make no allowance for irregularities in the operation of the process of pasteurization. The term "margin of safety" refers to the additional temperature and additional time necessary to provide an adequate margin, above any thermal death point of the tubercle bacillus, so that under no circumstances, in the routine operation of pasteurization, will the process fail to destroy the tubercle bacillus. It is obviously necessary in forming such an estimate to have reliable information as to the extent of the irregularities and fluctuations inherent in the pasteurizing process. It is to be regretted that such estimates in the past have not been based on scientific experimental data, but have been based for the most part on surmises and assumptions.

The pasteurizing apparatus, when properly made and supervised by competent operators, will confine the fluctuations in temperature within a fraction of one degree, and the period of time can be as accurately controlled as it can be under laboratory conditions. As a matter of fact, modern pasteurizing equipment, under automatic control in numerous pasteurizing plants, consistently controls both the temperature and the time even more accurately than can be done by hand operation in the laboratory. There is no margin of safety large enough to provide against the irregularities created by ignorance or dishonesty on the part of operators of such a process. If we consider, for example, the pasteurizing standard which had been adopted by New York City and fifteen other large cities, which is a minimum temperature of 142° F. for a minimum period of 30 minutes, an inspection of the chart shows that this temperature is 6° higher than the temperature of 136° at which the tubercle bacillus was killed at a period of 30 minutes in these experiments. The period of 30 minutes is 20 minutes more than is necessary to destroy the tubercle bacillus at 142° F. The city standards of 142° F. for 30 minutes then provide a margin of 6° in temperature and 20 minutes in time as a safeguard against accidental fluctuations in the pasteurizing process.

With modern equipment and competent operators the temperature can consistently be maintained between 143° and 142° F. with the time accurately controlled for 30 minutes. The 6° of temperature mentioned, therefore, provide an abundant margin of safety. Those who advocate the higher temperature of 145° for a period of 30 minutes do so on the assumption that the extra 3° is sufficient to cover the irregularities of imperfect equipment and incompetent operation. This is far from the truth since no pasteurization standard, even that of 212° is sufficient to provide against pasteurizing processes of this kind.

Another factor which has not rceived the attention it deserves has been pointed out by Professor Bang, who states that in feeding experiments with young rabbits the heating of milk for 2 minutes at a temperature of 140° was sufficient to weaken tubercle bacilli so that they could not infect rabbits through the alimentary canal.

It is obvious that there is a marked difference between the resistance of any animal to a hypodermic injection of tubercle bacilli, as compared with the feeding of tubercle bacilli. All of the tests made by hypodermic injection, as was done in these experiments, subject the laboratory animals to a far more severe strain than would be the case if the same dose of tubercle bacilli were administered with their food. The fact that the milk is not injected hypodermically into the tissues of milk consumers, but is taken by mouth, provides an additional margin of safety which is fairly indicated by the difference in resistance mentioned by Professor Bang. If, therefore, 2 minutes at 140° F. is sufficient to prevent infection when milk is introduced into the alimentary canal, a 30-minute period provides a margin of 28 minutes in time, which must be considered in estimating the margin of safety contained in any definition of pasteurization.

Conclusions

These are based both on the survey of the previous investigations and on studies of the operation of pasteurizing equipment and on the data reported herein.

1. Irregularities in the reports of various thermal death points of the tubercle bacillus are due primarily to faults in heating equipment and its operation by the investigators. These irregularities can be easily overcome by the use of simple heating equipment which can be properly operated by any intelligent operator.

2. There is no material difference in the resistance to heat between bovine and human strains of tubercle bacilli, and between different strains of either species, or between the tubercle bacilli in naturally infected milk, tissue lesions, or pure cultures.

3. Some of the work done by previous investigators was properly performed and furnished reliable data. These data indicate several thermal death points, as follows:

212° F. to 185° F. when heated for a few seconds
180° F. for 2 minutes
170° F. for 5 "
140° F. for 20 "

4. The volume of data reported in Bulletin No. 147 in the tests made at 142° F. and at 138° F. exceeds in quantity, by a large margin, the volume of data reported in any of the previous investigations, the results of which have already been accepted by bacteriologists. The care with which the work was performed and the authority of the investigators justifies the conclusions therein stated that tubercle bacilli are killed at 138° F. when heated for 30 minutes, and that a standard of 142° F. for 30 minutes is a proper standard for pasteurization.

5. There are an infinite number of thermal death points for the tubercle bacillus since for each period of time there is a degree of temperature at which this species of bacteria is destroyed. The number of tests sufficient to furnish statistical data shows that these thermal death points range themselves in regular order, the whole series, when plotted on a chart, forming a regular curve. This time-temperature relationship may be properly described as a "thermal death curve" rather than a "thermal death point."

6 If milk consumers were inoculated with hypodermic needles, as is done to guinea pigs, then the margin of safety of a pasteurizing standard of 142° F. for 30 minutes would provide for an excess of 6° in temperature and 20 minutes in time. In addition to this margin of safety it must be

recognized that milk consumers possess within themselves an additional safeguard against infections administered by the intestinal canal.

7. Temperature standards for pasteurization which injure the milk are not necessary for the protection of public health. The standard of 145° F., advocated by some authorities, changes the appearance of milk and thereby discredits pasteurization in the minds of milk consumers. It is in the interest of public health that pasteurization should be everywhere accepted. Its acceptance is prevented by such milk injuries. Standards used by New York City and fifteen other large cities for more than ten years have been proved by the experience of the health authorities of these cities satisfactory safeguards over public health, and at the same time have not injured the milk in a way that would prevent its acceptance by the public. These standards are a minimum temperature of 142° F. for a minimum time of 30 minutes. The results of the researches of previous investigators and of the researches herein reported show that this pasteurizing standard provides an adequate margin of safety.

For bibliography see the original article.

FURTHER STUDIES UPON THE COMPLEMENT FIXATION TEST IN CHRONIC GONORRHEA IN WOMEN

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M. A. Wilson, Mary V. Forbes and Florence Schwartz

These studies were undertaken in 1919 in coöperation with the Division of Venereal Diseases of the New York State Department of Health with the purpose of determining the value of the complement fixation test, as compared with other laboratory examinations, for the diagnosis of gonorrhea in women. The development of a clinical classification and improvements in method for smear, culture and complement fixation diagnoses were accomplished during the first year. The clinical classification and a comparison of diagnoses by smear, culture and complement fixation in 50 cases of mild, chronic gonorrhea in women were given in our preliminary report. Since then the time has been devoted to attempts to improve further the technic of the complement fixation test and to obtain an answer to the following questions: "Is the gonococcus complement fixation test specific?" "Is it an aid to the clinician in diagnosis?"

In attempting to answer the first question it is necessary to take up in detail the various factors in the test and, in discussing the possible defects, to show also the efforts that have been made to overcome some of these defects.

Although the second question is, in part, dealt with by tables 3 and 4, it is answered more fully in clinical papers published by Emily Dunning Barringer and E. von Bose and by Barringer and Williams.

Experiments to Improve the Gonococcus Complement Fixation Test

The technic of our diagnostic test was described briefly in our preliminary report (1) and later a detailed account of the method with interpretations of all the reactions was published in Pathogenic Microörganisms by Park and Williams. This method had been adopted by us as a standard before the present study was begun and has been continued as a standard, or control, in our attempts to improve the test.

Our experiments include:

Investigation of various methods for the preparation of antigen.

Tests as to the anticomplementary reaction of the patient's serum and the presence of natural antisheep amboceptor.

Standardization of complement.

Antigen

Many experiments have been undertaken by us to develop an antigen that will give a higher percentage of positive complement fixation reactions with serums of gonorrheal patients. These experiments dealt with the antigenicity of the gonococcus, the advantage of polyvalent antigens and the comparison of various culture media for antigen production.

Comparison of the antigenicity of the Torrey strains with recently isolated gonococci

In 1916 Dr. Archibald McNeil invited our coöperation in his study of antigen preparations. We isolated gonococci from 28 cases of acute urethritis in the male and made antigens of the new cultures at the same time and upon the same medium as the antigens prepared with the Torrey strains. Comparative tests of all the antigens were made with gonococcus immune rabbit serums and human gonorrheal serums. The freshly isolated cultures proved to be of lower antigenicity than the Torrey strains when tested with the same serums. The antigens from freshly isolated cultures had to be used in 1:10 dilution to obtain the same reactions that occurred with the Torrey strains in 1:20 dilution. The new isolations have less growth in the forty-eight hours than the Torrey strains, but the suspensions of the new cultures before being diluted and the suspensions of the Torrey strains before dilution were macroscopically of the same density, and all the antigen suspensions were made by weighing the dried cocci and adding saline in the proportion of 1 gram of dried cocci to 200 cc. of saline solution.

At the beginning of our present study, Miss Kutner isolated gonococci from 10 cases of gonorrhea and prepared an antigen from the cultures. These cases included both male and female adults.

Our complement fixation tests with her antigen were similar to those obtained with our antigens made from freshly isolated cultures.

In 1920 we isolated gonococci from 42 cases of gonorrheal vaginitis and made several polyvalent antigens. These, also, gave the same reactions in 1:10 dilution that the Torrey strain antigens gave in 1:20 dilution, thus, confirming the results of our two previous studies.

These experiments convinced us that the Torrey strains had not lost their antigenicity, and we have continued to use them for our stock antigens.

Will autogenous antigens and other monovalent antigens detect gonococcus antibodies that are missed by the Torrey strains?

Up to the present time we have given very little study to this subject, but are planning to follow it more fully in the future.

We have made three monovalent antigens: one from a culture isolated from acute gonorrheal vaginitis (Pecchio), one from a gonococcus isolated from Bartholin's gland (Dolson), and one from the blood of a scarlet fever patient having the complication of gonorrhea (Fazzio). The Dolson and Fazzio cultures were identified as gonococci by positive agglutination reactions in high dilution.

The agglutination tests were made by Dr. John C. Torrey.

The Pecchio antigen did not give a complement fixation reaction with the Pecchio human serum, but gave complete fixation, in 1:10 dilution, with a polyvalent serum from a rabbit immunized with the ten Torrey strains. The Pecchio human serum gave a weakly positive reaction with the Torrey polyvalent antigen. The Dolson monovalent antigen gave the same weakly positive reaction with its homologous serum as that serum gave with the Torrey polyvalent antigen and with the Fazzio monovalent antigen. These tests were made at the same time and with the same reagents. There was insufficient antigen prepared to continue the studies and the cultures were lost before another lot of antigen could be prepared. The following table shows these reactions:

TABLE 1.

COMPLEMENT FIXATION WITH MONOVALENT AND POLYVALENT ANTIGENS

Serums	Antigens			
	Monovalent			Polyvalent
	Pecchio dilution 1:10	Dolson dilution 1:10	Fazzio dilution 1:10	Torrey strains dilution 1:20
Pecchio (human)	Negative	No test	No test	Weakly positive
Dolson (human)	No test	Weakly positive	Weakly positive	Weakly positive
Fazzio (human)	No test	Strongly positive	Strongly positive	Strongly positive
Control. Rabbit immunized with Torrey's ten gonococcus strains	Strongly positive	Strongly positive	Strongly positive	Strongly positive

In the above tests it will be seen that the Pecchio serum did not react with its own antigen, but did react with the Torrey antigen.

The Dolson and Fazzio serums gave the same reactions with their own antigens that they did with the Torrey antigen. The rabbit serum (Torrey strains) reacted with all the antigens.

Effects of culture media upon antigenicity of the gonococcus

We have made polyvalent antigens with the ten Torrey strains upon various media, including salt-free-veal-agar, Vedder's starch medium, glucose-ascitic-veal-agar, vitamin-agar, glycerine-horse-serum-veal-agar, and disodium-phosphate-agar. Our findings are that the antigenicity of the gonococcus is not affected by the culture medium if the medium gives an abundant growth within forty-eight hours. Antigens made from twenty-four-hour, or seventy-two-hour growths, have to be used in lower dilution than those made from forty-eight-hour growths. However, although the antigenicity of the gonococcus is not affected by the culture medium, its anticomplementary property may be increased. We have found the antigens made from growth on Vedder's starch medium to be anticomplementary. Iodin tests revealed starch granules after the cocci had been washed many times to free them from the starch.[1] Media containing hemoglobin, also tend to produce an anticomplementary antigen. We have had uniformly good results with antigens made from growth on glycerine-horse-serum-veal-agar and on disodium-phosphate-agar. A small volume of antigen from growth on wheat medium was tried. There was not enough of the wheat medium antigen to test the human serums, but this antigen gave the same reaction with the gonococcus immune-rabbit-serum that the disodium-phosphate-agar antigen did.

We are planning a more complete study of different methods of antigen production.

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[1] The tests for starch were made by Dr. McNeil.

THE PATIENT'S SERUM

Two factors tend to give "false" reactions with the patient's serum: the anticomplementary reaction of the serum and the presence of natural antisheep amboceptor. The former gives a so-called false positive reaction, and the latter a false negative reaction.

Anticomplementary serums

In this laboratory we have had less than 1 per cent of anticomplementary patients' serums that could not be controlled by heating. The most notable example was a serum from a sixteen-year-old girl whose clinical diagnosis was "acute gonorrhea." This patient was bled at intervals of one week for thirteen weeks. The bleedings were tested in two laboratories and all reports were returned as "anticomplementary."

We tried a number of experiments to overcome this anticomplementary property. Upon the theory that the anticomplementary reaction in the patient's serum is due to the presence of both antigen and antibody in the right proportion to absorb complement, we tried to break down the antigen-antibody combination by adding either an excess of antigen, or an excess of antibody, but none of our experiments eliminated the anticomplementary property.

Active and inactivated serums gave the same anticomplementary reaction. The simple method recommended by Breuer was not tried on this serum, as his article had not been published at that time and his procedure had not been thought of by us. Breuer used a constant volume of patient's serum with varying amounts of complement and found that he could always obtain some dose of complement that would enable him to make a diagnosis of serums that would be anticomplementary with the usual dose of complement.

THE PRESENCE OF NATURAL ANTISHEEP AMBOCEPTOR IN THE PATIENT'S SERUM

The question of natural antisheep amboceptor is one to be considered. In table 2 are tabulated the results in a study of 2374 bleedings from 753 patients. The incidence of natural antisheep amboceptor that gave a negative reaction in a positive serum was only 0.3 per cent. In 60.6 per cent of these 2374 bleedings there was enough natural antisheep amboceptor to hemolyze from one-fourth to three-fourths of the sheep cells in the hemolytic system. In 50 per cent of the bleedings represented by the 60.6 per cent the small content of natural antisheep amboceptor did not interfere, apparently, with the complement fixation reaction. This conclusion was drawn from the fact that a human bleeding of one week might have no natural antisheep amboceptor and would give a weakly-positive reaction, while on the following week a bleeding from the same patient might have enough natural antisheep amboceptor to hemolyze half the cells and continue the weakly-positive reaction.

On the other hand, a number of serums having enough natural amboceptor completely to hemolyze all the cells in the hemolytic system have given a strongly positive reaction. In a recent example of this occurrence, the diagnostic test continued to be four-plus after standing in the water-bath for one hour, and then overnight in the ice-box.

As a control for natural antisheep amboceptor, we have used the Kaliski modification of the Bauer test, in which the natural antisheep amboceptor in the patients' serum is utilized for completing the hemolytic system, and, in the event of there being no antisheep amboceptor, or less than one unit, enough artificial amboceptor is added to produce complete hemolysis in the hemolytic system.

We have found the Kaliski method to be inadequate as a diagnostic test for the reason that a completely hemolyzed system is seldom obtained when artificial amboceptor is added to supplement the natural antisheep amboceptor.

The nature of the natural antisheep amboceptor reaction has not been explained sufficiently to enable one to control it absolutely. That an antigen in association with its homologous antibody can be dissociated from that antibody by the addition of an excess of artificial antisheep amboceptor is illustrated by the following example:

DISSOCIATION OF ANTIGEN FROM ITS HOMOLOGOUS ANTIBODY

First period of the test

Reagents	Diagnostic Test				Kaliski-Bauer Control			
	Serum control		Serum+antigen		Serum control		Serum+antigen	
Serum	0.04	0.02	0.02	0.01	0.04	0.02	0.02	0.01
Antigen	None	None	0.1	0.1	None	None	0.1	0.1
Complement	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Fixation period: One hour in water-bath at 37°C.

The foregoing chart shows the contents of all the tubes in the complement fixation tests up to the end of the fixation period. It will be noticed that the diagnostic test and the Kaliski-Bauer conrol contain exactly the same reagents in the same amounts. They are both fixed at the same temperature for the same time. Then, to the diagnostic test is added the standard dose of sensitized cells. (This dose contains 0.1 cc. of a 5 per cent suspension of sheep cells and two hemolytic units of antisheep amboceptor.) To the Kaliski-Bauer control is added 0.1 cc. of a 5 per cent suspension of sheep cells without any artificial amboceptor. After the cells have been added to all the tubes, the tests are replaced in the water-bath for the second incubation and at the end of ten minutes the reactions are read. The following chart shows the reactions in the tubes at the end of the second incubation period, after the addition of sensitized cells to the diagnostic test and 5 per cent suspension to the Kaliski-Bauer control:

Second period of the test After ten minutes in water-bath

	Diagnostic Test		Kaliski-Bauer Control	
	Serum control	Serum + antigen	Serum control	Serum + antigen
Reaction	Completely hemolyzed	Completely hemolyzed	Completely hemolyzed	Completely fixed
Diagnosis	Negative		Four-plus Interpreted as strongly positive	

As the above test and control were identical up to the end of the fixation period, if the complement were fixed as shown in the reading of the Kaliski-Bauer control, it must, also, have been fixed in the diagnostic test. Therefore, we conclude that the presence of natural antisheep amboceptor in the patient's serum did not interfere with the fixation of complement, but, that the addition of an excess of antisheep amboceptor dissociated the gonococcus fixation amboceptor in the patient's serum from the gonococcus antigen, thus, liberating the complement that had been completely fixed and giving a negative reaction. This point continues to be a subject for study in our laboratory.

The absorption of natural antisheep amboceptor has been recommended by some writers, but we have not found it to be an adequate remedy.

Table 2 shows the incidence of natural antisheep amboceptor in 2374 bleedings.

TABLE 2.

SHOWING THE INCIDENCE OF NATURAL ANTISHEEP AMBOCEPTOR IN PATIENTS' SERUMS

Number of Bleedings	Percentage Having One Unit, or More, of Natural Antisheep Amboceptor			Percentage Having less than one unit of natural antisheep-amboceptor	Percentage having no natural antisheep-amboceptor
	1.3				
	These are subdivided into:				
	Per cent positive reactions reduced to negative	Per cent positive reactions not changed	Per cent doubtful reactions		
2374	0.3	0.1	0.9	60.6	38.1

COMPLEMENT

The source of greatest difficulty in the performance of the complement fixation test lies in the variation of the guinea-pig complement. As we have shown in our preliminary report, the guinea-pig serums vary in fixability as well as in hemolytic activity, and before being pooled for tests all guinea-pig serums must be tested for natural antisheep amboceptor, hemolytic activity, anticomplementary reaction with the gonococcus antigen, anticomplementary reaction with the control gonococcus serum, and for fixability with the gonococcus antigen-serum-complex. The details of these preliminary tests and their interpretation have been published.

During the year 1920, out of 481 guinea-pigs tested, only 57 per cent could be used for gonococcus complement fixation.

HEMOLYTIC SYSTEM

After selecting the complement by the preliminary tests, the pooled complement is titrated with sensitized cells in order to determine the exact dose of pooled complement to be used for tests.

The sensitized cell dose contains two hemolytic units of antisheep amboceptor and 0.1 cc. of 5 per cent suspension of sheep cells.

The complement unit is read at the end of thirty minutes in the water-bath at 37° C. We use two hemolytic units of complement for diagnostic tests.

THE DIAGNOSTIC TEST

We use one-tenth the original Wassermann volumes of all reagents.

The diagnostic tests are made in duplicate, with controls for the hemolytic system, for anticomplementary reaction of the patient's serum, for natural antisheep amboceptor in the patient's serum, and for fixation unit and anticomplementary reaction of the gonococcus antigen.

The diagnostic test and all controls are fixed in the water-bath for one hour at 37°C.

Reading. The following controls must be completely hemolyzed before the diagnostic tests are read: system control, control for anticomplementary reaction in patient's serum, the antigen anticomplementary control (0.4 cc. of the antigen dilution must be completely hemolyzed, that is, four times the dose used in the test).

The average complement used throughout this study has given complete hemolysis in all controls in twelve minutes.

Diagnosis. If the patient's serum contains no natural antisheep amboceptor and if it is not anticomplementary, the diagnosis is made in accordance with the reactions in the tubes containing antigen plus serum. The Citron method for readings is used, with the exception of our "strong-one-plus," "one-plus" and "plus-minus" reactions, which Citron does not mention in his description of reactions.

Our "strong-one-plus" has very strong fixation in both the 0.02 and 0.01 tubes of the test; our "one-plus" has strong fixation in the 0.02 and weak fixation in the 0.01; our "plus-minus" has partial fixation in both the 0.02 and 0.01 tubes.

Anticomplementary serums. If the control for anticomplementary reaction in the patient's serum is not completely hemolyzed at the time required for hemolysis of the other hemolytic controls, the test is replaced in the water-bath until the end of one hour. If at that time the serum still shows any degree of anticomplementary reaction, no diagnosis is made of that bleeding, and it is reported as "anticomplementary."

Natural antisheep-amboceptor. If the diagnostic test is negative and the Kaliski-Bauer control shows a typical four-plus reaction, we report the test as: "Four plus by Kaliski-Bauer control." If the diagnostic test is negative and the Kaliski-Bauer control shows less than a four-plus reaction, we report the test as: "Negative. Serum contains an excess of natural antisheep amboceptor." In such cases we request another bleeding. If the serum controls in the Kaliski-Bauer control have partial hemolysis, we report the test according to the diagnostic test, regardless of the small amount of natural antisheep amboceptor. In table 2 we have shown that the presence of natural antisheep amboceptor reduced a positive reaction to negative in only 0.3 per cent of 2374 bleedings. Although this seems an insignificant number, we continue to use the control for the sake of the individual, and to give us an explanation of variation in the complement fixation curves of patients bled week after week.

Interpretation of diagnosis. All tests showing complete fixation in the 0.01 tube are interpreted as "very strongly positive." This is the Citron "four-plus" reaction, and is his interpretation.

All tests showing strong fixation in the 0.01 tube are interpreted as strongly positive. This includes Citron's "three-plus" and our "strong one-plus" reactions, and is Citron's interpretation of his "three-plus." In our "strong one-plus" reaction the 0.02 and 0.01 tubes are both very strongly fixed. Citron does not mention this reaction, but it is not infrequently encountered in the tests in this laboratory and must be considered in an accurate interpretation of diagnoses. It is stronger than Citron's "two-plus" and a shade less (in the 0.02 tube only) than Citron's "three-plus."[2]

All tests showing strong fixation in the 0.02 tube and weaker fixation in the 0.01 tube are interpreted as moderately positive. This is our "one-plus" reaction. It is stronger in the 0.01 tube than Citron's "two-plus," and a shade less in the 0.02 tube than his "two-plus." In our interpretation of moderately positive reactions, we include the Citron "two-plus." The latter is infrequently encountered in our tests, while our "one-plus" is of frequent occurrence. Citron does not describe our "one-plus."

All tests in which the 0.02 and the 0.01 tubes show partial fixation, (from 25 to 50 per cent hemolysis), are interpreted as weakly positive. This is our "plus-minus" reaction. It is stronger in the 0.01 tube than Citron's "two-plus" and has the same degree of fixation in the 0.02 tube as his "one-plus." This reaction is not described by Citron, but is of frequent occurrence in our tests.

In our weakly-positive interpretation we include Citron's "one-plus."

All tests showing 75 per cent. or more hemolysis in the 0.02 tube are interpreted as "doubtful."

Our interpretation is the result of the study of a large number of tests in relation to the clinical classification.

In cases clinically diagnosed as negative for gonococcus we have obtained no fixation, not even a doubtful reaction, and for that reason we feel justified in placing in the positive column the reaction described above.

SPECIFICITY OF THE GONOCOCCUS COMPLEMENT FIXATION TEST

In this study we have examined repeated bleedings from 870 gonorrheal cases controlled by bleedings from 350 non-gonorrheal individuals. In testing the specificity of the gonococcus antibody 92 of the gonorrheal bleedings were tested with tuberculosis antigen and gave negative reactions, and 54 of the gonorrheal bleedings were tested with *Streptococcus viridans* antigen and gave negative reactions. We have not included the Wassermann antigen in our controls of specificity of antibody for the reason that a considerable number of the patients in this service have a clinical diagnosis of syphilis, or doubtful for syphilis, as well as of gonorrhea.

In testing the specificity of the gonococcus antigen, bleedings from 350 patients clinically diagnosed as non-gonorrheal gave no trace of gonococcus complement fixation reaction.

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[2] Unpublished report of the Research Laboratory Committee on Standardization of the Wassermann Test.

Table 3 shows the incidence of gonococcus complement fixation at various stages of the disease and also the specificity tests upon non-gonorrheal serums

TABLE 3

GONOCOCCUS COMPLEMENT FIXATION IN VARIOUS STAGES OF GONORRHOEA IN WOMEN

CLINICAL DIAGNOSIS	NUMBER OF CASES	GONOCOCCUS COMPLEMENT FIXATION			
		Positive	Negative	Doubtful	Percentage positive
Acute gonorrhea.*	19	9	6	4	47
Subacute gonorrhea	112	54	36	22	48
Chronic gonorrhea	383	283	56	44	73 6
Syphilis and gonorrhea	162	132	14	16	81.4
Syphilis, doubtful for gonorrhea	113	24	88	1	21
Doubtful gonorrhea	81	49	21	11	60
Controls					
Adults having no symptoms or history of Gonorrhea	50	0	50	0	0
Children: Scarlet fever patients having no symptoms or history of gonorrhea	36	0	36	0	0
Spinal fluids from poliomyelitis cases	12	0	12	0	0
Adults: Tubercular patients having no gonorrheal symptoms	248	0	248	0	0
Normal bleedings from workers in this laboratory	4	0	4	0	0
Total cases	1220				

*Not *early* acute.

Table 4 shows the comparison of smear, culture and complement fixation in 181 cases. These three laboratory tests are more fully discussed in a paper by Torrey and Wilson.

TABLE 4.

COMPARISON OF DIAGNOSES BY SMEAR, CULTURE AND COMPLEMENT FIXATION

CLINICAL DIAGNOSIS	NUMBER OF CASES	GONOCOCCUS COMPLEMENT FIXATION											
		Smear positive, culture positive			Smear doubtful, culture positive			Smear doubtful, culture negative			Smear negative, culture negative		
		Positive	Negative	Doubtful	Positive	Negative	Doubtful	Positive	Negative	Doubtful	Positive	Negative	Doubtful
Acute	11	3	2	1	1			1	2				1
Subacute	43	10	1	3	6	1		9	4	3		4	2
Chronic	85	9	1	1	2			39	2	8	15	2	6
Chronic and syphilis	35	3		1				16	2	5	7	1	
Doubtful	7							2	3		1	1	
Total cases	181												

In table 5 is given a summary of the diagnoses reported in table 4, showing how the percentage of positive diagnoses by smear and culture decrease with the stage of the disease and how the percentage of positive complement fixation increases.

TABLE 5.

SUMMARY OF DIAGNOSES

Clinical Diagnosis	Number of Cases	Percentage of Positive Diagnosis		
		Smear	Culture	Complement fixation
Acute gonorrhea*........................	11	55	55	45
Subacute gonorrhea......................	45	32	44	58
Chronic gonorrhea.......................	85	13	15	76
Gonorrhea and syphilis..................	35	11	11	76
Doubtful gonorrhea......................	7	None	None	42
Total cases.............................	181			

* Not *early* acute.

SUMMARY

Investigation of various media for the preparation of gonococcus antigen showed that any of the starch-free and hemoglobin-free media that would give a profuse growth of the gonococcus within forty-eight hours at 37°C. would give antigens of equal value.

Recently isolated gonococci were of lower antigenicity than the Torrey strains isolated many years ago.

Autogenous antigens gave no stronger reactions with their homologous serums than did the Torrey strain antigen.

In this study we have encountered very few anticomplementary patients' serums that could not be controlled by heating.

The incidence of natural antisheep amboceptor that gave a negative reaction in a positive serum was only 0.3 per cent of 2374 bleedings. We found the Kaliski-Bauer test to be inadequate as a diagnostic measure, but of value as a control. Absorption of natural antisheep amboceptor did not give us uniform reactions.

As previously reported, we found the difference in fixative and hemolytic value of guinea-pig complements to be the greatest factor of variation in the gonococcus complement fixation test.

In order to give a true interpretation of our diagnostic tests in relation to the clinical classification, we have had to include three reactions frequently encountered in our tests that were not mentioned by Citron. Otherwise, our interpretations agree with Citron's.

Specificity tests with heterologous antigens gave no fixation with gonorrheal serums, as shown in table 3.

Non-gonorrheal serums tested with gonococcus antigens gave no complement fixation.

Tables 4 and 5 show the relative value of smear, culture and complement fixation. These three laboratory methods are discussed in a paper by Torrey and Wilson.

The clinical value of our gonococcus complement fixation tests is discussed in the publication of Barringer and Williams, and Barringer and von Bose.

CONCLUSIONS

1. Individual guinea-pig-serums must be tested for fixability with known gonococcus antibody plus gonococcus antigen before they can be used as complement for tests of patients' serums.

2. The negative results with control human serums throughout this series of tests indicate that the gonococcus complement fixation tests is specific.

3. The gonococcus complement fixation test is an aid to the clinician in institutional work.

For bibliography see the original article.

A STUDY OF THE KAHN PRECIPITATION METHOD FOR THE SERUM DIAGNOSIS OF SYPHILIS

Published in The American Journal of Syphilis, 1928, Vol. XII, pp. 111-121.

By M. A. Wilson, Rose M. Nedley and Florence Honig

This study covers a period of two years, during which time 22,030 serums (3,424 cases) were tested by the Kahn precipitation test in comparison with the Wassermann test.

The authors desire to express their appreciation of the cooperation in this investigation of Dr. Louis Chargin, Chief of the Division of Venereal Diseases, who selected cases for our study; of Mr. John Koopman, Bacteriologist in charge of the Wassermann Laboratory, who gave us serums for Kahn tests and reports of his Wassermann diagnoses of those serums, as well as giving us space for three technicians in his laboratory; of Dr. David Kalisky, of Mt. Sinai Hospital, who sent us serums, clinical data, reports of his Wassermann tests, and also sent his technician to our laboratory to make comparison tests and readings with our technicians.

We are particularly indebted to Dr. E. von Bose, of Kingston Avenue Hospital, for her kindness in sending us clinical data which enabled us to make classified tables for our report.

The precipitation tests were made by Kahns' latest method.[1] Dr. Kahn very kindly gave us personal instructions in regard to all the technic of the test.

Mr. Koopman's Wassermann tests were made with the crude alcoholic antigen, only, by the New York City Department of Health qualitative method.[2]

In the series of serums sent from the Venereal Disease Clinics, the results were summarized, as follows:

Medical Advice Clinic. 118 cases, one bleeding from each case. Agreement in diagnosis 85%.

By the term, "agreement in diagnosis," is meant that both tests were positive (i. e., 4, 3, or 2 plus), or doubtful, (+), or negative.

This term corresponds to Kahn's term, "absolute agreement," as used in his latest publication.[3]

Advice Clinic. 1503 cases, one bleeding from each case. Agreement in diagnosis, 85.5%.

Manhattan Clinic. (Treatment Cases.) 915 cases having repeated bleedings.

The *case diagnosis* (i.e., the curve of repeated bleedings) by Kahn test and Wassermann test agreed in 49% of the cases. Variation in *case diagnosis,* 51% of the cases. Two thirds of the variations can be explained as occurring at the beginning or end of the serologic test curve. These variations ranged in degree of reaction from $\left\{\begin{array}{l}\text{Kahn four-plus}\\ \text{Wassermann negative}\end{array}\right.$ to $\left\{\begin{array}{l}\text{Kahn negative}\\ \text{Wassermann four-plus}\end{array}\right.$
In Table I we have shown several examples of such variations.

TABLE 1.

SERUM NUMBER		COMPARATIVE TESTS.				EXPLANATION OF VARIATIONS IN THE WASSERMANN AND KAHN TESTS
1	Wass.	—	—			Beginning of complement fixation curve.
	Kahn	3	4			
2	Wass.	—	—	—		" " " " "
	Kahn	±	±	2		
3	Wass	—	—	—		" " " " "
	Kahn	±	+	3		
4	Wass	—	±			" " " " "
	Kahn	+	2			
5	Wass.	—	—	±		" " " " "
	Kahn	±	—	+		
6	Wass.	—	±	—	±	End of complement fixation curve.
	Kahn	2	+	+	±	
7	Wass.	±	—	—		" " " " "
	Kahn	2	±	—		
8	Wass.	±	—			" " " " "
	Kahn	2	+			
9	Wass.	—	—	—		" " " " "
	Kahn	4	4	2		
10	Wass.	—	—	—		" " " " "
	Kahn	3	2	+		
11	Wass.	—	—	—		" " " " "
	Kahn	4	2	+		
12	Wass.	±	—	±	—	" " " " "
	Kahn	4	4	3	+	

For the remaining one-third of the variations in case diagnosis we find no explanation in these tests in the absence of clinical data.

As we were given no clinical diagnoses for the clinic cases, we are unable to make classified charts of those tests for this paper.

From Kingston Avenue Hospital we received blood serums from 788 cases, with a total of 3,359 repeated bleedings. Most of the bleedings were made at intervals of one week during the period one patient was in the hospital. Those tests may be summarized, as follows:

Those cases diagnosed as clinical syphilis had complete agreement in the *case diagnosis* by Kahn and Wassermann tests. By the term, "case diagnosis" is meant that throughout the curve of repeated bleedings from the patient one or more of the bleedings had the same diagnosis by Kahn and Wassermann. Under *Positive* case diagnosis are listed all the serums that were four-plus, three-plus, or two-plus by Kahn and Wassermann. Under *doubtful* case diagnosis are listed all serums that were one plus by Wassermann and Kahn. Under *negative* case diagnosis are listed all serums that were negative, or plus-minus, by Wassermann and Kahn. We placed the plus-minus reactions with the negatives instead of with the doubtfuls for the reason that usually the clinician interprets a plus-minus reaction as negative.

In cases clinically diagnosed as "Serologic Syphilis," or as "Syphilis and Gonorrhoea," there were variations in the case diagnosis by Kahn test and Wassermann test, as follows:

Serologic Syphilis:
39 cases, agreement in case diagnosis 70%.
Variations: Kahn stronger than Wasserman 20%.
Wassermann stronger than Kahn 10%.

By "Serologic Syphilis" is meant cases having no clinical symptoms of syphilis, but having a positive Wassermann test when admitted to the hospital.

Syphilis (not classified as to stage of disease) 213 cases. 185 of these were complicated with gonorrhoea and had agreement in case diagnosis in 95.5%, and the remaining 28 cases, not complicated with gonorrhoea had agreement of 57%.

Gonorrhoea, subacute:
24 cases, agreement in case diagnosis 91.5%.
Both tests positive 8.5% of the cases.
Both tests negative 83% of the cases.
Total variations: Kahn stronger in 8.5% of the cases.

Gonorrhoea, chronic:
130 cases, agreement in case diagnosis 93.5%.
Both negative 88%.
Both positive 5.5%.
Variations: Kahn stronger, 5.5%.
Wassermann stronger, 1%.

Gonorrhoea, clinical:
145 cases, agreement 88%.
Both negative 78.5%.
Both positive 9%.
Both doubtful 0.5%.
Variations: Kahn stronger, 7%.
Wassermann stronger 5%.

By "gonorrhoea clinical" is meant cases having clinical symptoms of gonorrhoea and all smears negative.

The data from which the above summary was made are shown in Table II.

TABLE 2.

SHOWING AGREEMENT OF KAHN AND WASSERMANN CASE DIAGNOSES AND RELATION OF THOSE CASE DIAGNOSES TO THE CLINICAL DIAGNOSIS.

Clinical Diagnosis	Total Cases	Percentage of Agreement of Kahn and Wassermann Diagnoses	Agreement.			Variation	
			Kahn and Wassermann Diagnosis Agreed Throughout Curve, or at some time during the Curve.			Wass. Stronger than Kahn	Kahn Stronger than Wass.
			Number of Cases Whose Case Diagnoses were:				
			Pos.	Neg.	Doubt.		
Syphilis, Primary	1	100%	1				
Syphilis, Active Secondary	7	100%	6	1			
Syphilis, Non-Active	7	100%	6	1			
Syphilis, Serologic	39	70%	24	3		3	9
Syphilis, Not Classified as to Stage of Disease	28	57%	16			1	11
Syphilis and Gonorrhoea	185	95.5%	159	19			15
Gonorrhoea, Subacute	24	91.5%	2	20			2
Gonorrhoea, Chronic	130	93.5%	7	115		1	7
Gonorrhoea, Clinical	145	88%	13	114	1	6	10

NOTE: 198 cases of gonorrhoea, not classified as to stage of disease, and 22 cases from which no clinical data were received, are not included in this table.

In order to determine the exact agreement of Wassermann and Kahn tests of individual serums, the tests described as case-diagnosis, or "curve-diagnoses" in Table 2, were listed to show the number of bleedings that were four-plus by both Wassermann and Kahn, three-plus by both, etc. This list is shown in Table 3.

Table 3 is summarized as follows:

Primary syphilis, 1 case, had 6 bleedings, as follows:
 1 bleeding was plus-minus by both tests.
 1 was four-plus by Wassermann and three-plus by Kahn
 3 were four-plus by Wassermann and two-plus by Kahn
 1 was two-plus by Wassermann and negative by Kahn

Syphilis:
 Active secondary, 7 cases, 31 bleedings.
 16 bleedings were four-plus both tests
 2 bleedings were negative both tests

Syphilis:
 Non-Active, 7 cases 23 bleedings
 6 bleedings were four-plus both tests
 4 bleedings were negative both tests

Syphilis, Serologic:
 39 cases, 172 bleedings
 61 bleedings were four-plus both tests
 5 were three-plus both tests
 4 were two-plus both tests
 1 was plus both tests
 1 was plus-minus both tests
 10 were negative both tests

Gonorrhoea subacute:
 22 cases, 105 tests
 4 bleedings were four-plus both tests
 97 bleedings were negative both tests.

Gonorrhoea chronic:
 130 cases, 557 bleedings
 10 bleedings were four-plus both tests
 2 bleedings were two-plus both tests
 1 bleeding was plus-minus both tests
 515 bleedings were negative both tests

By comparing the arrangements of the same tests in Tables II and III it is shown that the percentage of agreement of the two tests will be smaller when the comparison is made upon the identical diagnosis of the same bleeding by both tests, than when the comparison is made upon the interpretations positive, negative and doubtful.

We have not found in the literature any report of a study of repeated bleedings from the same patient, and we thought it might be of interest to include in our publication a table showing the Kahn and Wassermann curves of repeated bleedings. From the total 788 cases, we have selected from each clinical classification a few curves that are fairly representative of the whole. These are shown in Table IV under the heading, "Comparative Tests," each column represents a specimen of blood tested by Wassermann and Kahn methods. The numerals in each column represent the diagnosis reported to the clinician expressed in terms of 4, 3 or 2 plus, etc.

TABLE 4

Clinical Diagnosis	Case Number	Test No.	Comparative Tests.												
			1	2	3	4	5	6	7	8	9	10	11	12	13
Syphilis. Primary	1	Wass.	4	4	4	4	±	2							
		Kahn	2	2	3	2	±	—							
Syphilis. Active.....		Wass	—	±											
Secondary	2	Kahn.	—	—											
	3	Wass.	4	4	4										
		Kahn.	4	4	4										
	4	Wass.	2	4	4	+	±								
		Kahn.	4	4	4	4	3								
	5	Wass.	4	4	3	+	—	+							
		Kahn.	4	4	4	4	4	4							
	6	Wass. Kahn.	4 3	4 4	4 4	4 4	4 4								
	7	Wass.	4	4	4	4	±	+	+						
		Kahn.	4	4	4	4	4	4	4						
	8	Wass.	4	±	—										
		Kahn.	4	4	3										
Syphilis. Latent.	9	Wass.	4	4	2	±									
Act. Sec.		Kahn.	4	4	4	4									
	10	Wass.	+	±	+	—	2	—							
		Kahn	3	4	4	4	4	2							
	11	Wass.	4	4	4										
		Kahn.	3	3	3										
Syphilis. Serologic.	12	Wass.	4	4	4	3	4	4	4	4	4	3			
		Kahn.	4	4	4	4	4	4	4	4	4	4			
	13	Wass.	4	4	4	4	2	—							
		Kahn.	4	3	4	4	4	4							
	14	Wass.	4	4	4	±	3	+							
		Kahn.	2	4	4	4	3	4							
	15	Wass.	2	—	—	—	2	2							
		Kahn.	2	+	+	+	2	—							
	16	Wass.	4	±	+	—	—								
		Kahn.	3	3	±	2	3								
Syphilis and Gonorrhoea	17	Wass.	—	—	—	2	4	3	2	4	4	3	+	±	±
		Kahn.	—	—	±	3	4	4	2	4	3	4	4	4	4
	18	Wass.	±	—	—	+	2	±	—	—	2	—	±	2	+
		Kahn.	—	3	2	2	3	4	2	2	2	2	2	3	2
	19	Wass.	4	4	4	4	4	4	4	4	+	±	+		
		Kahn.	3	3	+	3	4	4	3	4	±	4	4		
Gonorrhoea. Subacute	20	Wass.	—	—	—	—	—	—	—	—	—	—	—	—	
		Kahn.	—	—	—	—	—	—	—	—	—	—	—	—	
	21	Wass	—	—	—	—	—	—	—	—	—	—	—	—	
Chronic		Kahn.	—	—	—	—	—	—	—	—	—	—	—	—	
Chronic	22	Wass.	—	—	—	—	—	—	—	—	—	—	—	—	—
		Kahn.	—	—	—	—	—	—	—	—	—	—	—	—	—
Clinical	23	Wass.	—	—	—	—	—	—	—	—					
		Kahn.	—	—	—	—	—	—	—	—					

Summary

22,030 serums were tested by the Kahn precipitation test and by the New York City Department of Health Wassermann method.

In single bleedings from 1,621 advisory cases from the Venereal Diseases Clinics the Kahn and Wassermann tests agreed in diagnosis, as positive, doubtful, or negative, in 85% of those tests.

In repeated bleedings from 915 cases undergoing treatment, the case diagnosis agreed in only 49% of the cases. Of the 51% variations of 62% of them could be explained as being at the beginning or ending of a curve, while for 38% of the variations we could find no explanation in the absence of clinical data.

In repeated bleedings from 788 cases at Kingston Avenue Hospital (3,359 bleedings) for which we received clinical data, the Kahn and Wassermann case-diagnoses agreed in 100% of those cases clinically diagnosed as active syphilis.

In serologic Syphilis, by which is meant cases having no clinical symptoms of syphilis, but having a positive Wassermann reaction, the agreement of the Wassermann and Kahn case-diagnoses was only 70%. The syphilitic cases were all under treatment.

In syphilis and gonorrhoea, not classified as to stage of either disease, the agreement was 95.5%.

In gonorrhoea not complicated by syphilis the agreement of Kahn and Wassermann was less than in the syphilitic cases, but was greater than in the unclassified list of tests of specimens from the Venereal Disease Clinics.

A close examination of the Wassermann and Kahn curves of 788 cases, of which an excerpt is given in our Table IV, shows the variations in the Wasserman tests throughout the curves and the Kahn tests throughout the curves to be approximately the same. When the Wassermann test curve was irregular the Kahn was usually irregular. The irregularities occurred more often in the latter stages of disease.

In the attempt to evaluate these comparative tests, we must keep in mind the fact that the Kahn tests were made with an antigen very strongly reinforced with cholesterin, (0.6%), whereas, the Wassermann tests were made with a crude alcoholic extract antigen containing no cholesterin. Thus, the Kahn tests had the advantage of a much more sensitive antigen. Had the Wassermann tests been made with the Kahn antigen, the variations in the treated cases might have been less. Our percentage of agreement of diagnosis, positive, negative and doubtful, is greater than Kahn's in his latest report.[3]

Conclusions

Our study shows a strong similarity between the Kahn and Wassermann tests, regardless of the fact that the Kahn antigen was more sensitive.

Most of the variations of single tests could be explained upon the fact of the more sensitive Kahn test detecting antibody earlier in the curve and later in the curve. A small percentage of tests in which the Wassermann test was positive and the Kahn test negative was not explained by any of the tests in our study.

Our tables show the difficulties in drawing accurate conclusions in regard to the relation to stage of disease of Wassermann and Kahn tests when single bleedings from treated cases are considered. Ones should have repeated bleedings over a period.

References:
[1] Kahn, Serum Diagnosis of Syphilis, 1925 ed.
[2] Park & Williams, Pathogenic Microorganisms, 1924 ed., p. 278.
[3] Kahn, Jour. A. M. A., 1927, Vol. 89, No. 2.

THE LIMITS OF ACCURACY OF HEMOLYTIC TITRATIONS

John Koopman

The precise titration of the hemolytic reagents is a matter of fundamental importance in complement fixation work.

The fact that it is so difficult to obtain agreement among several laboratories in the results of Wassermann tests is due in a large measure to complement variations.

The unit of complement as determined by the customary titrations is a variable and indefinite quantity. It is not uniform in all laboratories and it is not uniform in any one laboratory from day to day.

As the result of an effort to increase the accuracy of complement titrations two methods of titrating complement have been devised. Although both of these methods are accurate, they may still produce different results from the same sample of complement. Because of this fact it is possible to show that titrations alone cannot be depended upon to control complement fixation tests, so that the results will be free from variations.

One of the reasons why the complement unit as at present determined is not constant is due to the fact that we cannot control the density of the cell suspension.

In order to show how variations in the density of the cell suspension produce a variable complement unit it is only necessary to prepare a simple titration such as the following:

Place a unit of complement and an excess of amboceptor, say two units, in each of seven or eight tubes. Add graded amounts of cells to these tubes so that the first will receive less than the customary amount and the last more than the customary amount with intermediate amounts in between. Balance with saline so that all tubes contain the same volume and incubate. Several tubes will probably show complete hemolysis and several incomplete hemolysis. All of these tubes contain exactly the same amount of complement and amboceptor; notwithstanding this fact it will be noted that whether a tube shows complete hemolysis depends upon the amount of cells present rather than upon the amount of complement.

This fact has been universally recognized and because the complement unit is based upon the hemolysis of a definite quantity of cells, numerous attempts have been made to standardize the cell suspension.

There is no practical method of preparing cell suspensions which can be guaranteed to produce uniform results in the hands of different workers. In fact it is very difficult for any one laboratory to control the uniformity of its own cell suspension from day to day.

The various methods which have been proposed to overcome this difficulty will not be discussed here, because the following procedure will show that the error caused by variations in the cell suspension would not be overcome even if it were possible to prepare a uniform cell suspension from day to day.

By means of a 1 c.c. pipette graduated in .1 c.c. place .1 c.c. of 5% cells in each of 10 tubes. Add an equal amount of water to each tube so as to hemolyze the cells. If the resulting solutions in the 10 tubes are compared in a colorimeter the results will show that the amount of cells delivered by each division varies. This variation is due to two factors, one is the unavoidable technical error and the other is the sendimentation of the cells in the pipette.

The last tenth c.c. contains fewer cells than any other tenth and the slower the pipetting is done the greater will be the variations between the first and the last tenth. This being so, it becomes necessary to make a change in the method of titrating complement so that the error caused by these unavoidable cells suspension variations will be automatically eliminated.

It was thought at one time that the error due to variations in the cell suspension was neutralized in complement fixation tests if the same cell suspension was used for the test as was used for the titration of the complement. This is not so because if a weak cell suspension is used in the titration it will require less complement to bring about complete hemolysis. Under these circumstances the unit will be smaller and less complement will be used in the fixation reaction itself. Therefore a weakly positive serum which fixes a small amount of complement may nevertheless fix all the complement. It would therefore be reported as giving a stronger reaction than it is entitled to. On the other hand if a denser cell suspension is used to titrate complement, more complement will be added to the fixation tests and therefore some strongly positive specimens may be reported as weakly positive.

A method of estimating complement strength which gives constant results regardless of cell suspension variations may be demonstrated as follows:

If two suspensions of sensitized cells are prepared one of which would be considered slightly less and the other slightly greater than 5% we will have two suspensions which represent the maximum variation which is likely to occur from day to day or which may occur in different laboratories.

Prepare two complement titrations in the customary manner and use the light cells in one and the heavy cells in the other. At the end of one hour's incubation it will be found that the hemolytic unit will vary in the two titrations.

The heavier cells will require slightly more complement to produce complement hemolysis.

Allow the cells in the tubes which show incomplete hemolysis to settle and compare the hemolysis in these tubes. It will be found that the corresponding tubes in each titration will show the same amount of hemolysis. The amount of cells added to one set was greater than the other and the amount of cells remaining is greater in one set than the other but the amount of hemolysis is unaffected.

With the aid of these titrations we can form an estimate as to the relative vlaue of the information furnished by an incompletely hemolyzed tube as compared with a completely hemolyzed tube.

When hemolysis proceeds until all the cells present in a tube have given up their hemoglobin it is correct to say that the reaction is complete. It is complete however only as regards the cells because there may be present in the solution some complement and amboceptor which would produce more hemolysis if more cells were present to be acted upon.

The titration above shows that complete hemolysis may be brought about in several ways. A tube containing a little more complement and more cells and another containing less complement and fewer cells will both show complete hemolysis and thus indicate a false unit. In addition to the variation of the unit caused by the cell variation another difficulty is raised when we try to determine whether hemolysis is just complete or not quite complete. All that can be said of a completely hemolyzed tube is that the reagents present are sufficient to hemolyze the cells present. The actual amount of either cannot be definitely determined.

An incompletely hemolyzed tube gives much more definite information.

A tube which contains a small amount of complement in the presence of an excess of amboceptor and cells will not hemolyze completely. Under these circumstances the reaction comes to an end only when the complement has produced all the hemolysis it is able to produce. Therefore if we stop using the point of complete hemolysis as a guide for the complement unit and use the actual amount of hemolysis produced as our index of complement strength, one of the sources of error will be eliminated.

The above method of estimating complement strength is an excellent one when it is desired to compare two complements for their relative activity. When it is used to determine the absolute strength of an unknown complement however it exhibits a serious discrepancy.

There is no universal permanent standard which can be used from day to day or place to place to show how much hemolysis a definite amount of complement should produce. Under these circumstances it would be necessary to depend upon the eye alone in judging whether the complement produced too little or too much hemolysis. This would be introducing as great an error as existed in the first place.

Therefore an addition must be made to the above method so that any degree of hemolysis may be expressed in terms having the same significance to all workers, notwithstanding the fact that no inter-laboratory comparison is possible.

The following titrations lead up to such a method of indicating complement strength.

Place .05 c.c of 10% complement in each of 6 tubes. To the first tube add .25 c.c. of salt solution, to the second .3 and so on to the last tube which receives .5 c.c. Add to each tube .1 c.c. of 5% cells and .15 c.c. of amboceptor.

We now have 6 tubes which contain the same amount of all reagents, the only difference being in the volume employed.

Incubate these tubes until the reaction comes to an end and centrifuge so that the amount of hemolysis in each may be compared.

It will be seen that the amount of hemolysis produced by .05 c.c. of complement depends upon the volume in which it acts. The amount of hemolysis in the first tube will be greater than that in the last tube, just as if different amounts of complement were present. This is partly due to the fact that the color is confined to a smaller volume in the first tube, but if the volume in all the tubes is now made uniform with salt solution it will be found that the graduations in color are still present.

Continuing with above kind of titration it will be seen that by varying the volume between still wider limits, it is possible to make .05 c.c. of complement produce any amount of hemolysis desired.

With a sufficiently large total volume, .05 c.c. of complement produces no hemolysis whatever. With a very small volume the hemolysis is so active that it will be necessary to use a heavier cell suspension if the complement is to show its full effect before all the cells are used up.

This being so it is evident that the amount of hemolysis produced by complement does not depend upon the actual amount of complement present but upon its concentration. This fact has an important bearing on the titration of complement.

Quantitative hemolysis takes place in accordance with the laws of mass action. This fact permits the activity of any complement to be accurately determined and the result stated in terms of definite significance.

As a result of a series of tests made with various concentrations of the same complement the following figures were obtained. The readings were made with a colorimeter.

Two dilutions were made from the same sample of complement, one 10% and the other 8%. A comparison was made of the amount of hemolysis produced by an equal quantity of each dilution. .2 c.c. of the 10% complement produced twice as much hemolysis as did .2 c.c. of the 8% complement, the total volume in each tube being 4 c.c.

This shows that the two complement dilutions which differ by 25% in actual concentration may differ by 100% in the amount of hemolysis they produce.

Likewise, if the concentration of any complement is doubled, the amount of hemolysis it is then able to produce is multiplied by three or four or some greater number.

There are two ways in which the concentration of complement may be varied, one is by varying the amount of complement in a given volume of solution and the other by using a given amount of complement and varying the volume of solution in which it acts.

The extent to which the amount of hemolysis is changed when the concentration of complement is raised or lowered may be used as a guide to the strength of the complement. For instance, using the customary 10% complement, .2 c.c. will probably produce 5 times as much hemolysis as .1 c.c in a total volume of 4 cc. That is, doubling the concentration increases the hemolysis 5 times.

If we prepare a weaker solution of this complement by making an 8% dilution and then repeat the above test we find that .2 c.c. of this weaker dilution may produce 6 times as much hemolysis as .1 c.c. in a volume of 4 c.c. That is, doubling the concentration of the weaker complement changes the amount of hemolysis 6 times whereas doubling the stronger complement changes the hemolysis 5 times.

If we make the complement still stronger by preparing a 12% dilution from the original stock, the hemolysis will only be increased 4 fold if we double the concentration by using a .2 c.c. and .1 c.c. in the same volume.

The amount by which the hemolysis is increased when the concentration is doubled varies very nearly inversely as the strength of the complement.

That is if we have at hand two samples of complement of unknown activity one of which produces 6 times as much hemolysis when its concentration is doubled and the other produces 3 times as much when its concentration is doubled, then the latter sample is for all practical purposes twice as strong as the former or as 3 is to 6.

In any given volume of solution and two given amounts of complement, there is but one strength of complement which will produce a given difference of hemolysis.

Therefore any complement solution which shows an agreed difference of hemolytic activity when its concentration is altered by an agreed amount has an agreed titre.

If the above statements are true, the activity of any complement may be stated in figures having the same significance to all workers and complement of any desired hemolytic activity may be prepared at will.

The relation of volume to hemolysis is worthy of further study. A reason for the fact that the amount of hemolysis varies with the concentration of the complement and not with the actual amount used, can be deduced from the following titration.

Prepare a series of 8 tubes, in the first tube put .06 c.c. of 10% complement, increase the amount by .02 c.c. until the last tube receives .20 c.c. Add sufficient salt solution to make the volume in each tube 2.5 c.c. Then add sufficient sensitized cells to make the volume 5 c.c. in each tube and incubate to completion of the reaction.

Remove the cells which remain unhemolyzed and compare the color of the solution in the several tubes by means of a colorimeter.

A titration of a certain complement made in this manner gave the following readings:

Amount of complement........	.06	.08	.10	.12	.14	.16	.18	.20 cc.
Colorimeter reading	none	none	30	15	10	7.5	6	5

Inasmuch as the colorimeter gives a smaller figure as the color increases, the figures must be reversed so that increasing hemolysis will be represented by increasing numbers. When this is done the relative amount of hemolysis in the tubes is as follows:

Complement	.06	.08	.10	.12	.14	.16	.18	.20 cc.
Hemolysis	0	0	5	10	15	20	25	30

It will be seen that .2 c.c. of complement gave a color of 30 which is 6 times as much as was produced by .1 c.c. It will also be seen that the difference in hemolysis for each .02 c.c. of complement is a constant of 5. If each reduction of .02 c.c. of complement changes the hemolysis by 5, then a reduction of .02 c.c. from the .1 c.c. tube should reduce the hemolysis from 5 to zero.

That this reasoning is correct is shown by the fact that .08 c.c. of complement did not produce any hemolysis whatever.

This means that .08 c.c. of this complement was used up in some manner without producing any hemolysis. It also means that regardless of how much of this complement was present in any tube the first .08 c.c. of it did not produce any hemolysis. Whatever hemolysis is produced in any tube is the result of the amount of complement which is in excess of .08 c.c. The tube containing .1 c.c. of this complement therefore has only .02 c.c. available for hemolysis. The same thing holds true for the other tubes as follows:

Total complement added..........	.06	.08	.10	.12	.14	.16	.18	.20
Available for hemolysis..........	0	0	.02	.04	.06	.08	.10	.12
Amount of hemolysis produced...	0	0	5	10	15	20	25	30

The amount of hemolysis produced is in direct proportion to the amount of complement available for hemolysis and not to the total amount present.

If we apply this method to any complement it will be found that by a comparison of the amount of hemolysis produced in any two tubes it is possible to calculate the activity of the complement. An example of the calculation is as follows:

In a volume of 5 c.c. .1 c.c. of complement produced a color of 5 and .16 c.c. produced a color of 15.

Let A represent the large amount of complement
Let B represent the small amount of complement
Let X represent the greater hemolysis complement
Let Y represent the smaller hemolysis complement

$$B - \frac{(A-B)\,Y}{X-Y} = \text{The concentration of the complement}$$

Substituting figures for the 2 tubes of the above titration we find

$$.1 - \frac{(.16 - .1)5}{15-5} = .07$$

The figure obtained by this calculation is a significant one for the following reason. It will be found that it always represents the largest amount of complement which can be added to sensitized cells without producing any hemolysis. This figure varies directly as the actual strength of the complement.

Two complements which give 7 and 8 as a result of such a test stand in this relation to each other in actual strength.

Sensitized cells seem to require a definite concentration of complement before hemolysis begins.

If the concentration of complement in a tube is less than the required minimum no hemolysis takes place although the actual amount of complement present may be a considerable one.

If the concentration is increased it will finally reach a point where the cell resistance will be overcome and hemolysis will begin.

This required concentration of complement being a constant, the activity of any complement may be determined by finding how much or how little of it can be added to a definite volume of solution before hemolysis begin.

Any complement can be brought to an agreed standard strength by changing its dilution until a definite amount in a definite volume will just do this.

In order to titrate complement by this method it is only necessary to prepare a 10% dilution of the unknown and set up six tubes as follows:

Complement	.01	0.15	.02	.025	.03	.035 cc.
Salt solution to make the volume in all tubes...	.3 cc.					
Sensitized cells	.2	.2	.2	.2	.2	.2 cc.

The largest amount of complement which produces no hemolysis is a direct indication as to the activity of the complement.

Experience has shown that six times this amount corresponds to the amount of complement we aim to use when we use 2 units as determined by the classical method of titrating.

This method far surpasses the classical titration method in accuracy for several reasons.

1. The only tube in a titration which varies exactly as the strength of the complement is the tube which shows no hemolysis.

2. It is much easier to determine a point of no hemolysis than it is to determine the point of complete hemolysis.

3. When complete hemolysis is read the number of cells present makes a difference in the result. This is not the case when the point of no hemolysis is read.

Additional reasons in favor of using the point of no hemolysis as a guide are the following:

Colorimeter readings will show that when any tube shows complete or almost complete hemolysis of a fairly dense cell suspension such as is used in ordinary titrations, relatively large increases or decreases in the amount of complement will produce relatively small changes in the amount of hemolysis. This is if we use a strongly hemolyzed tube as a standard by which to judge complement strength, large variations in the amount of complement may pass unnoticed because they produce such small changes in the amount of hemolysis. This is a very important cause of error in judging the ordinary complement unit.

If we judge the amount of complement by the amount of hemolysis in a tube which is only partly hemolyzed or not at all hemolyzed the conditions are entirely different, in this case a very slight increase or decrease in the amount of complement will produce a magnified difference in the amount of hemolysis. The chances for accuracy are therefore all on the side of the method which uses slightly hemolyzed or unhemolyzed tubes as guides.

A simple method of showing this to be so is to add .1 c.c. and .15 c.c. of complement to two tubes. Add .75 c.c. of sensitized cells and .65 c.c. of salt to each.

The hemolysis will be almost complete and both tubes will be very nearly alike, as to color. Using the same amount of complement and sensitized cells in two duplicate tubes add 3.5 c.c. of salt to each incubate and compare the color. Because of the larger volume the second pair of tubes will show little hemolysis but the difference between them will be great. When the hemolysis is great, 50% difference in the amount of complement produces about 20% difference in the amount of hemolysis. When the hemolysis is small 50% difference in the amount of complement makes more than 100% difference in the amount of hemolysis.

It was stated in the beginning of this article that several methods for accurately titrating complement would be given and that their very accuracy could be used to show that titrations alone cannot be depended upon to produce accurate fixation results.

The above method is one of these and the reason it does not guarantee accurate fixation results is as follows:

If a sample of complement is titrated with the cells of several sheep it will be noticed that occasionally the titre will show a marked variation with the cells of some one sheep. This variation will also occur when fresh cells are compared with cells which are several days old even though they are from the same sheep.

The cells of different sheep vary markedly in their resistance to the action of the hemolytic reagents. If we adjust the complement in accordance with the cells of each sheep a different amount of complement might be used in each instance.

Under these circumstances although the above method corrects one error peculiar to the customary titration and that is the error due to differences in cell suspension density, it still retains the error due to variations in cell resistance.

If the cells are less resistant than normal, the complement will appear stronger than it really is because of the increased hemolysis. If the strength of the complement is reduced and used in a fixation reaction, each specimen will receive less complement than usual and less complement need be fixed in order for the test to show complete fixation.

Any method of estimating complement strength which depends upon the amount of hemolysis produced will always be subject to this type of error. Increasing the accuracy of such titrations does not help us to determine the actual amount of fixable complement.

A Titration of Actual Complement Strength

This method provides for the estimation of complement strength in terms of fixable units regardless of the fact that such a complement may produce more or less hemolysis with cells of different sheep or cells of varying resistance.

Of all the reagents used in hemolytic work, amboceptor is the most stable. It retains its titre over relatively long periods of time. For this reason if a definite dilution of a particular amboceptor is made daily it furnishes a standard which can be used to determine how much the other reagents vary. If, for example, .1 c.c. of a 1 to 1,000 dilution of a certain amboceptor produces more hemolysis one day than another we know that the difference is due to the other reagents and not to the amboceptor. The difference may be due to the complement used or to the cells or to a combination of both.

If that part of the difference which is due to the variation in complement strength can be subtracted from the total the remaining difference will be due to the cells alone.

The variation in hemolysis which is due to variation in complement strength can be neutralized or allowed for.

The following titrations will show this to be so.

The Quantitative Relationship of Complement to Ambocepter

Variations in the strength of the ambocepter used in complement titrations and variation in the strength of the complement used in amboceptor titrations can be prevented from affecting the results of such titrations if the time factor in reading is removed.

That is, it makes no difference whether the amboceptor used to titrate complement varies considerably if the titration is read at the end of its reaction time instead of after a definite number of minutes.

To illustrate this point prepare two complement titrations which are identical except that one is completed with a dilution of amboceptor which is considered fast and one with a dilution which is considered slow. If these titrations are read after ten minutes incubation they will read differently because of the difference in amboceptor. After 20 minutes the difference will be less but still considerable. If they are read after both have reached the point where hemolysis ceases, the results will be absolutely alike. The end point for one may be 45 minutes and for the other 75 minutes or more.

If the conditions are reversed and amboceptor is used in small amounts in the presence of an excess of complement the same thing may be said of complement as was said of amboceptor in the above titration.

As a result of the information furnished by these titrations the following statement is justified.

Variation in the excess of amboceptor in a complement titration or variation in the excess of complement in an amboceptor titration changes the time required for the reaction to reach completion. It does not change the end point. This means that in titrating an unknown complement, any variation in the strength of the amboceptor such as was used above or such as may occur in different laboratories will have no affect on the result provided that a reading is taken when the action has ceased and not at the end of any definite number of minutes. Furthermore the result can be accepted with greater certainty if the excess of amboceptor is reasonably large and the time correspondingly short, making the end point more definite.

With any combination of complement and amboceptor, the amount of possible hemolysis is limited by the reagent which is present in the lesser amount while the time is controlled by the one present in the greater amount.

THE COMPENSATION OF AMBOCEPTOR FOR COMPLEMENT

If we prepare two tubes, one containing a small amount of complement, say .02 c.c. with an excess of amboceptor say .07 c.c. and the other containing amboceptor say .02 c.c. and an excess of complement say .07 c.c., they may both show the same amount of hemolysis.

This fact has led to the erroneous impression that a lack of one reagent is compensated for by an excess of the other.

The reason these two tubes show an equal amount of hemolysis is that .02 c.c. of complement can only do a certain amount of work regardless of the amount of amboceptor present and when its work is done any amboceptor in excess of the amount required by the complement remains in the tube. The same is true of .02 c.c. of amboceptor which is able to do a definite amount of work and when this is done any excess of free complement is left in solution. The equal amount of hemolysis produced is not an evidence of compensation.

If a lack of complement was compensated for by an excess of amboceptor we would be able to make .05 cc. of complement produce as much hemolysis as .07 c.c. of complement by adding more amboceptor to .05 c.c. of complement.

The amount of hemolysis which a definite amount of complement is able to produce cannot be increased by adding more amboceptor. It can be decreased by adding an insufficient amount of amboceptor, but in this case the hemolysis is limited to the amount which the amboceptor can produce and the complement is then in excess.

Another fact concerning the relationship of complement and amboceptor is that neither reagent is able to produce its maximum hemolysis unless there is present an excess of the other. One reason for this is the change in the reaction time caused by an excess of either reagent. The smaller the excess the greater the time necessary for the reaction to reach completion and when there is no excess of either the time is infinite and a small amount of each reagent remains unused. This explains why a unit of a complement plus a unit of amboceptor does not produce complete hemolysis although either one can do so if an excess of the other is present.

There is no danger of a complement titration hemolyzing too fast or too far as the result of an excess of amboceptor, in fact the end point is most easily determined when the excess is reasonably large and the time short.

We know that if an excess of complement is used in the presence of a small amount of amboceptor, the actual strength of the complement does not affect the amount of hemolysis produced by the amboceptor, but alters the reaction time only.

It must follow therefore that if the amboceptor is kept at a constant dilution and the complement, cells and time factor are in excess, any variation in the amount of hemolysis from day to day is due only to the resistance of the cells.

Advantage may be taken of these facts to titrate complement to an exact fixable standard. We can adjust the strength of the complement by means of the constant amboceptor and thus discount the variation in the resistance of the cells.

An example of such titration is the following:

Choose a dilution of amboceptor which will contain 2 units in each .1 c.c. and consider this dilution arbitrarily as a standard.

.05 c.c. of such a dilution is allowed to hemolyze with .15 c.c. of 10% complement, and .2 c.c. of cells. The final volume in the tube should be made up to 1 c.c. with saline. This tube will vary in hemolysis from day to day only if the cells vary in resistance and for no other reason. Variations in complement strength do not affect it.

Prepare a 10% dilution of the complement to be titrated and place various amounts in a series of tubes as follows:

.035 .040 .045 .050 .060 cc.

Add an excess of amboceptor and cells and equalize with salt to a volume of 1 c.c. Incubate these tubes and the amboceptor tube mentioned above, to the completion of the reaction. Centrifuge all of the tubes. The color of the solution in the amboceptor tube is compared with the color in the complement tubes.

The tubes containing various amounts of complement will produce an amount of hemolysis which, as has been explained above, will depend upon the activity of the complement modified by the resistance of the cells.

Some one of these tubes will produce an amount of hemolysis which will be the same as the amount of hemolysis produced in the amboceptor tube.

The amount of hemolysis in the amboceptor tube may vary with the resistance of the cells, but whatever hemolysis does take place is always produced by the constant amount of amboceptor. The complement tube which produces the same amount of hemolysis as the amboceptor tube is also affected by the cell resistance to the same degree. This tube however contains ,a standard amount of complement because the same cells resistance is common to all the tubes and we have compared the complement directly with the stable amboceptor.

Under this plan the fixable amount of complement can be accurately determined regardless of the fact that it may produce more or less hemolysis, with different cell suspensions.

Either of the two methods given above for the titration of complement will produce accurate results from its own point of view but each method may produce a different result from the same sample of complement.

From a consideration of these titrations it is evident that complement may be titrated for its hemolytic value or for its actual fixable quantity, but neither method will guarantee accurate fixation results.

If we add to our complement fixation tests that amount of complement which produces a definite amount of hemolysis we may be adding different fixable units.

If we add a standard fixable amount of complement to the tests regardless of the amount of hemolysis it is able to produce, the cells of some particular sheep may cause the amount left for hemolysis to appear greater or less than it really is and thus lead to false conclusions as to the strength of the reaction.

OSMOSTIC PRESSURE AND ITS RELATION TO SPECIFIC AND NON-SPECIFIC HEMOLYSIS

John Koopman

The fact that small changes in the tonicity of the hemolytic reagents cause a considerable change in the amount of hemolysis they produce, led us to investigate the role of osmosis in specific and nonspecific hemolysis.

Water added to a asuspension of erythrocytes causes them to lose their hemoglobin. Exactly what happens to the cell itself is not clearly understood.

Recent text books on physiology state that the cell takes up water and increases in volume until it finally bursts from the effect of the increased internal pressure.

The results of a number of simple experiments with cell suspensions and water seem to indicate that the cells do not swell up and do not burst but remain intact during and after hemolysis.

If we prepare a number of salt solutions, starting with .8% and decrease the percentage by .025% until we reach .525%, their effect on sheep cells will provide data of some interest.

Place 1 c.c. of each solution in a series of twelve tubes. To each tube add a small amount of a rather heavy suspension of cells in serum or in isotonic salt solution. .05 c.c. or 1 drop of the cell suspension will be sufficient to produce the effect desired. Hemolysis will probably take place to a very slight extent in the .75% or the .7% tube. Each succeeding tube will show a larger amount and the .525% tube may show complete hemolysis.

After standing about one-half hour the cells and salt solution will have reached their equilibrium and no further hemolysis will take place. The remaining cells will settle.

If we hold to that explanation of hemolysis which supposes the cells to burst when they give up their hemoglobin under reduced pressure, we must say that in the first tube some of the cells have burst and some have remained intact. In each succeeding tube a still larger number of cells must have burst until all have been destroyed.

If some cells are presumed to be more fragile than others we must believe that there is a gradation in fragility among cells which causes them to break up in regular order under uniformly reduced pressure. This of course is not possible.

Proceeding with the above experiment, the supernatant fluid in each of the tubes is poured off after the cells have settled. Divide the fluid from each tube into two parts and keep one set of tubes for control. To each tube of one set add another drop of the original suspension of cells. These new cells will again partly hemolyze to almost the same extent as the first cells did and will almost double the color of the fluid. That is, partial hemolysis again takes place in the tubes which could not hemolyze any more of the first cells added.

A reasonable explanation of what has taken place is this: When cells are added to a hypotonic salt solution each cell promptly adjusts itself to the new condition by dialyzing hemoglobin until the pressure within the cell has fallen to that of the fluid in which it is suspended. This means that if a small amount of cells is added to a hypotonic salt solution, the solution can not take from these cells enough hemoglobin to increase its tonicity to normal but only enough to reduce the cells to the tonicity of the solution. When the cells have lost enough hemoglobin to reduce their internal pressure to that of the surrounding medium, no further hemolysis can take place although the fluid surrounding them is still hypotonic. In order for the fluid to withdraw more hemoglobin more normal cells must be added. These in turn will only give up enough hemoglobin to adjust themselves to the new tonicity of the salt solution.

Within certain limits, the greater the number of cells added the deeper will be the color of the solution because a greater number of cells must lose some of their hemoglobin in adjusting themselves to the new condition. This loss of hemoglobin will take place upon each addition of normal cells until the hemoglobin released has raised the tonicity of the fluid to that of a normal cell.

Although the cells may have lost part of their hemoglobin there has been no rupture or other injury to them. This will be found to be so if the cells remaining in any tube are resuspended in salt solution of normal tonicity; they will then act exactly like normal cells. In the case of complete hemolysis in water each cell loses all its hemoglobin before reaching equilibrium with the external fluid.

The adjustment of a cell to its surroundings takes place very quickly. It takes place in a very short time when the pressure of the fluid outside is greater than that of the hemoglobin inside, but when the pressure outside is smaller the adjustment is exceedingly rapid. A simple experiment will illustrate this.

If cells suspended in normal salt solution are added to a salt solution two or three times normal they will float because they are so much lighter, but in a very few minutes they will have made the necessary exchange and begin to settle just as they do in normal saline and at about the same rate.

If on the other hand cells are suspended in 3% salt solution and an amount of water is added which will just dilute that solution to normal, it is almost impossible to mix the two without some of the cells hemolyzing.

When the fact is taken into consideration that dilute salt solutions such as these are known to reach equilibrium with almost incredable rapidity when mixed, it will be realized how quickly the cell must react in order to hemolyze before the stronger salt solution mixes with the water.

If cells suspended in strong salt solution are poured into another solution of normal tonicity which has been made by mixing the 3% salt solution with enough water to bring it to normal, no hemolysis takes place. This shows that it is not the shock of being poured into a solution of lower tonicity which causes the hemolysis, but the fact that the cells are affected by the water before the stronger salt can diffuse into it and raise it to normal.

Osmotic Pressure and Specific Hemolysis

The sheep which we keep on hand to supply us with blood for our Wassermann work, sometimes develop a varying resistance to our specific reagents. This variation among different sheep formerly caused considerable trouble in our complement titrations and fixation work. Complement kept frozen from day to day would sometimes appear to have become much stronger or much weaker because of the varying resistance of the cells of the several sheep. Upon numbering the sheep and keeping records we were finally able to predict just what effect each sheep would have upon the titre of complement and thus avoid surprises due to the apparent change of strength of our reagents.

On making tests of each sheep's blood with salt solutions of varying tonicity as outlined above, we found that the cells which showed some hemolysis with .775% salt solution also hemolyzed rapidly with our hemolytic reagents and made the complement appear to be very active. Cells which showed no hemolysis until the salt solution was reduced to .7 or less also resisted hemolysis with the hemolytic reagents and caused the complement to appear weaker.

Because of this apparent relation between osmotic pressure and specific hemolysis, the effect of salt solutions of various percentages upon specific hemolysis was investigated. It was found that the difference between the amount of hemolysis obtained by using all reagents made up with .9% salt as compared with .8% salt is considerable, and that the amount of hemolysis can be controlled by altering the tonicity of the salt solution.

If it is possible to change the amount of hemolysis produced by the hemolytic reagents by altering the tonicity of the salt solution osmosis must play a large part in specific hemolysis as well as in water hemolysis.

When partial hemolysis is produced by the hemolytic reagents we have no more reason for saying that some of the cells have broken up and some have not than we have for saying this when hypotonic salt solution produces the same effect. Dialysis of hemoglobin seems to be the controlling factor in both. Cells which are partly hemolyzed specifically may be resuspended in stronger salt solution and they will show no visible injury.

If the hemolysis produced by the hemolytic reagents is due to osmosis where has the change in pressure taken place? The presence of complement and amboceptor does not reduce the tonicity of the salt solution because if it did, the erythrocytes of any animal would hemolyze with any amboceptor. The presence of complement and a specific amboceptor must therefore raise the internal pressure of the erythrocytes. The cell then dialyzes hemoglobin until the internal pressure falls to normal again.

If we add .05 c.c. of 10% complement to sensitized cells in the presence of salt solutions of increasing tonicity from .9% up, the first tube will probably show complete hemolysis. Each succeeding tube will show a smaller amount of hemolysis until a point is reached where no hemolysis takes place. The series will look exactly like a titration when varying amounts of complement are used. In fact complement may be titrated with perfectly satisfactory results by altering the salt solution instead of complement.

The condition may also be reversed and varying amounts of complement added to tubes containing salt solution of double physilogical strength. The largest amount of complement which can be added without producing any hemolysis will be an index of its potency.

We have found that it requires a salt solution of somewhere near 1.7% to keep .05 c.c. of 10% complement from showing any hemolysis of sensitized cells in a total volume of .5 c.c. This means that this amount of the hemolytic reagents has caused an increase of 100% in the interial pressure of the cell, because any outside pressure less than that of double strength salt will allow the hemoglobin to diffuse.

In the experiments outlined above where partial hemolysis was produced by hypotonic salt solutions, it was shown that hemolysis ceases when the tonicity of the cell has fallen to that of the surrounding fluid. The fluid is still hypotonic and is able to withdraw more hemoglobin if more cells of normal tonicity are added.

The same conditions seem to govern the amount of hemolysis produced by the specific hemolytic reagents. Assume that we have a tube which contains sufficient complement and sensitized cells to produce only partial hemolysis after prolonged incubation. It may appear that the remaining cells would hemolyze if any complement remained and that the only reason the hemolysis ceases is because the complement is exhausted.

It can be shown that the complement is not exhausted and that more hemolysis will occur if more sensitized cells are added. The additional cells cannot of course be added to the same tube. Two tubes must be prepared to show this effect, each one receives the same amount of complement. One tube then receives a certain amount of salt solution and sensitized cells, the other receives a double dose of sensitized cells and a correspondingly smaller amount of salt solution in order to keep the volume in each tube equal.

The tube containing the larger amount of cells will show the larger amount of hemolysis. An explanation of the cause of this increase is as follows.

The complement present is able to raise the internal pressure of a cell a certain amount.

The amount by which it is raised depends upon the concentration of the complement.

Although the complement present cannot raise the pressure of this cell high enough to produce complete hemolysis it still can raise the internal pressure of more normal cells to the same extent. When this is done more hemolysis is produced because more cells lose an equivalent amount of hemoglobin in returning to normal pressure. Just how far this increase of hemolysis with increase of cells will go was not determined.

The information to be gained from a comparison of the physics of hemolysis by hypotonic salt solutions and by specific reagents is this: Regardless of the nature of the changes which occur within the cell when it is placed in contact with amboceptor and complement, the hemolysis which takes place after that is due to an increase of the osmotic pressure within the cell. The amount of hemolysis which occurs is in direct proportion to the increase in the internal pressure. When we speak of partial hemolysis we do not mean that a few of the cells have hemolyzed completely and others not at all but that all of the cells have given up a portion of their hemoglobin to the same degree.

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